Investigation of the Proteinpolysaccharides of some Connective Tissues.

A thesis submitted for the degree of

Doctor of Philosophy

in the

Australian National University

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February 1968

This thesis embodies the results of work carried out in the department of Physical Biochemistry, John Curtin School of Medical Research during the period between January 1965 and December 1967. In accordance with the regulations of the Australian National University I wish to state that the work was carried out entirely by myself, except where other - wise stated in the text and that work which is described in chapter 3, where I worked in collaboration with Dr. J.R.Dunstone.

Maureen D. Franek.

Maureen Diana Franek.

It is a pleasure to acknowledge the advice and encouragement of Dr. J.R.Dunstone under whose supervision this work was carried out.

I would like to thank Professor A.G.Ogston F.R.S. for his continued interest and invaluable comments throughout the course of this work.

I am indebted to Dr. D.S.Shaw and Mr. L.B.James for carrying out amino acid analyses, to Dr. E.Spinner for the determination of infrared spectra and to Mr. I.Reid for the running of polyacrylamide gel electrophoreses.

The assistance of Mr. R.A.Goodman, Head Technician of the Department of Physical Biochemistry in procuring apparatus and chemicals and in very many other matters is very much appreciated. I would like also to thank Mr. D.Hardman of the Department of Genetics and Dr.A.J.Gibbs of the Department of Microbiology for making available to me their departmental ultracentrifuges.

I wish to express my thanks to the management and staff of the Canberra Abattoirs for their willing co - operation when I was collecting biological material.

To Mrs. K.Hopper and Miss F.Richardson for their patience in the typing of this thesis and to Mr. R.Weston for advice when compiling the photographic sections of this work I am sincerely indebted.

Finally,I am grateful for the award of an Australian National University Research Scholarship,during the tenure of which,the work described here was carried out.

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CHAPTER I.

General Introduction

I:I. INTRODUCTION.

Connective tissue is distributed widely throughout the body in tendons, sheaths, some subcutaneous tissues, supporting tissue of organs and in cartilage. It is a mesenchymal tissue, containing at least four main constituents, namely, cells, vessels, fibres and ground substance.

The realization that such tissue is important in a number of physiological and pathological reactions has stimulated increased investigational efforts into its structure and functions. In particular many such studies have been directed toward the examination of the ground substance, in the belief that this substance is of prime importance in the proper physiological functioning of the tissue.

Complexes of protein and polysaccharide are important constituents of this ground substance and many functions have been ascribed to them. In many instances, the inferences are largely speculative, despite an abundant literature of their structure, distribution, properties and metabolism. In particular Fessler (1960), and Partington and Wood (1963) have emphasized the probable importance of these compounds in determining the tensile properties of connective tissue.

Ogston and Phelps (1960), Laurent and Ogston (1963), Laurent (1963, 1964) and Gerber and Schubert (1964) have considered, in some detail, the effects of carbohydrate and non-collagen protein-polysaccharide polymers on the hydration and excluded-volume properties of such tissues.

More recently, Milch (1966) has proposed that ground substance polysaccharides, which appear to undergo very characteristic alterations in a number of age and disease related clinical states, may also act as compatible plasticizers or polymer diluents for native collagen chains.

In some tissues, for example, aorta, the ground substance contains a wide spectrum of different proteinpolysaccharide materials (Buddecke and Schubert, 1961; Meyer, 1964; Radhakrishnamurthy, Fishkin, Hubbell and Berenson, 1964). In other tissues such as cartilage only a small number of different protein-polysaccharides are present (Buddecke, Kroz and Lanka, 1963; Marler and Davidson, 1965; Luscombe and Phelps, 1967a; Muir and Jacobs, 1967). For this reason and also due to its ready availability, cartilage has been used frequently in investigations of the structure and functions of the proteinpolysaccharides present in

connective tissue. However, the results obtained in such instances cannot be assumed to apply to similar material present in other tissues or in other mammalian species, unless careful studies of the properties of these materials, performed by identical or closely similar means, have shown that such comparisons are justified.

The work described in this thesis has aimed to compare the physicochemical characteristics, as measured in isolated systems, of proteinpolysaccharides from nasal cartilage with similar preparations from aorta. However, in order that the functions of these proteinpolysaccharides may be considered in true perspective, a knowledge of the physical and chemical nature of the ground substance in which they occur, is required. The subsequent parts of this introductory section will therefore include, a brief anatomical description of the nature of connective tissue, a review pertaining to the present knowledge of the macromolecular structure of proteinpolysaccharides and finally an account of the lines along which this research has been directed.

I:2. CONNECTIVE TISSUE

Connective tissue is usually conceived as containing relatively few cells per unit volume when compared with highly cellular organizations such as liver; additionally, it can be differentiated from the latter by the presence of a significantly greater quantity of extracellular material. Further, connective tissue contains an organised fibrillar network composed variously of the biochemically distinct fibrous proteins, collagen, elastin and reticulin. These are embedded in an amorphous medium termed the ground substance.

I:2:I. THE CELLULAR COMPONENTS OF CONNECTIVE TISSUE.

Of the various cell types present in connective tissue, fibroblasts, macrophages and mast cells are the most common. Fibroblasts, including their specialized derivatives, such as chondroblasts and osteoblasts have been clearly linked to the formation of collagen and ground substance (Dorfman, 1959). The mast cells are also thought to be implicated in the formation of proteinpolysaccharides; they are known to contain and to synthesize heparin (Schiller and Dorfman, 1959). The macrophages have phagocytic properties; they appear to play the part of scavengers, removing

cell debris and particulate matter from the connective tissue (LeGros Clark, 1945).

I:2:2. THE EXTRACELLULAR COMPONENTS OF CONNECTIVE TISSUE.

The extracellular material of connective tissue is made up of the fibrous proteins, collagen, elastin and reticulin embedded in an amorphous matrix, loosely described as ground substance.

I:2:2:I. The Fibrous Proteins.

(i). Collagen is a fibrous protein, occurring in wide straight unbranched white bundles, that possess high tensile strength and low elasticity (McKusik, 1966). A characteristic feature of this protein is the amino acid profile. About 33 percent of the amino acid residues are glycine and a further 25 percent are the amino acids, proline and amino hydroxyproline (Gross, 1964).

The collagen fibrils are composed of long, rigid, rod-shaped molecules approximately 2800 A in length and 14 A in diameter (Boedter and Doty, 1955). They display a characteristic 640 A periodicity, as observed by small angle X-ray diffraction and electron microscopy (see Harrington and von Hipple, 1961). This periodicity bears a direct correlation to the amino-acid sequence (Grassman, 1966); i.e. to alterations of

apolar regions, rich in proline and hydroxyproline, and polar regions, poor in imino acids but rich in basic and acidic amino acids.

Each molecule is composed of three polypeptide chains, one of which, (α-2), differs in amino acid composition from the other two, (α-1) (Piez, Eigner and Lewis, 1963). These chains are coiled in a left-handed helix, and wound about each other in a rope-like fashion in the right-handed direction (see Gross, 1964).

(ii). The protein, elastin, occurs in two main forms (Jackson and Cleary, 1967): (1), as fibre bundles as in ligament, the adventitia of muscular arteries and elastic cartilage, and (2) as fenestrated sheets or lamellae, as in the walls of aorta and the main blood vessels.

On a molecular level, elastin is believed to be composed of chains which adopt a randomly-kinked conformation and are cross-linked at intervals by firm chemical cross bonds (Partridge, 1966a). Such a structure implies that the protein chains are kinetically free and not engaged in any kind of crystalline configuration so that, under the action of thermal motion, the system contracts (Partridge, 1966a).

Desmosine and isodesmosine are believed to be important agents in the cross-linking (Partridge, 1964). More recently, LaBella, Keeley, Vivian and Thornhill (1967) have found evidence which suggests that dityrosine might be involved in the linkage region.

Elastin is further characterized by a very unusual amino acid composition, in which over 90 percent of the residues have non-polar side chains and nearly 18 percent of these residues are valine (Partridge, 1964).

(iii). Reticulin is seen in the electron microscope as a feltwork of fine randomly arranged fibrils, varying in diameter from 100 to 600 A and lying in an apparently amorphous matrix (Melcher, 1966).

Reticulin fibres have somewhat similar properties to collagen fibres, the most important being the 640 A periodicity (Kramer, 1952). The amino acid profile is also similar to that of collagen (Piez and Likins, 1960), with probably a smaller amount of proline and a larger amount of hydroxyproline.

On the basis of electron microscope observations, and on biochemical, X-ray diffraction and histochemical evidence it has been suggested that reticulin could comprise collagen in varying states of aggregation (Melcher, 1966).

I:2:2:2. The Ground Substance.

The ground substance is the extracellular, extrafibrillar, amorphous matrix of connective tissue. It has a varying consistency and is composed of salts, water, protein and polysaccharides. It has become increasingly evident that the polysaccharides are present in the tissue in chemical combination with non-collagenous protein (Partridge and Davis, 1958; Malawista and Schubert, 1958; Buddecke et al., 1963; Luscombe and Phelps, 1967a).

The polysaccharides are high molecular weight anionic glycosaminoglycan* or glycosaminoglycuronoglycan* polymers. The amount of protein associated with these carbohydrate polymers is still uncertain. However two principal types may be distinguished. Those containing about 80 percent protein and which can be compared with glycoprotein materials isolated from other sources (Radhakrishnamurthy et al., 1964; Eylar, 1965) and those containing only 20 to 30 percent protein, for example the uronic acid-containing protein isolated from bovine nasal cartilage (Gerber, Franklin and Schubert, 1960).

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* For nomenclature see section I:3:I.

I:2:3. CARTILAGE.

Cartilage is a specialized form of supporting connective tissue. The cellular components are disposed in discrete groups of three, four or more and are distributed throughout the extracellular matrix enclosed in capsules.

The matrix is translucent and almost structureless, but it can incorporate varying quantities of ordinary connective tissue fibres. If only a few fibres are present, the cells and their matrix are called hyaline cartilage, if there are many collagen fibres in the matrix, the name fibrocartilage is applied, and if elastin fibres predominate the tissue is called elastic cartilage (Sinclair, 1966).

In the adult mammal, hyaline cartilage is found on the ventral ends of ribs, on the surface of bones within joints and in the respiratory passages. It is more widespread in the embryo (Bloom and Fawcett, 1962). Elastic cartilage is rare; it is found in cartilages such as are found in the mobile part of the nose and the external ear. Fibro-cartilage is more widely distributed and forms such important structures as the intervertebral discs and the intra-articular cartilages found in many joints (Sinclair, 1966).

Cartilage in the joints has the property of sustaining great weights and at the same time allowing the bones which carry this weight to move easily and smoothly against one another. In other places, such as the ear and in the respiratory passages, cartilage serves as a pliable yet resistant framework which prevents the collapse of the passages. Finally, the cartilage of many bones makes possible their growth in length and is important in determining their size and shape (Bloom and Fawcett, 1962).

Thus, far from being an inert tissue, cartilage, through its participation in the growth of bones, can be a fairly delicate indicator of certain metabolic disturbances; it reflects nutritional deficiencies, especially those involving proteins, minerals and vitamins (Bloom and Fawcett, 1962).

I:2:4. CONNECTIVE TISSUE OF THE ARTERIAL SYSTEM.

The arterial system constitutes, in principle, simply a conduit mechanism whereby the products of the ventricular overflow tract of the heart are distributed to the body cells and tissues. The arteries undergo a considerable variety of apparently inexorable "degradative" changes almost immediately upon cessation of their growth and development (Milch,

1965).

The arterial wall is composed essentially of three layers, the tunica adventitia, the tunica media and the tunica intima, the last being a continuous, extremely delicate lining of longitudinally disposed flattened endothelial cells (Milch, 1965). Besides the endothelium, the arteries are composed of fibroblasts and collagen fibres, bands and networks of elastin and smooth muscle cells (Bloom and Fawcett, 1962).

The arteries are classified according to their size into three categories, those of a large calibre or elastic type, in which the middle layer is distinctly yellow due to the predominance of elastic elements, those of a muscular (or distributing) type, where the middle layer is red-grey because of the smooth muscle cells and finally, the very small arteries or arthérioles, which are 0.3 mm or less in diameter.

The aorta is the largest artery and leads directly out of the heart and thus has to take the full impact of blood forced out by its contraction. For this reason the aorta contains a large amount of elastic tissue in its walls.

The composition of the other ground substance components of aorta are as yet far from clear.

Considerable confusion and frequently glaring inconsistencies are evident even with respect to the composition of the polysaccharide phase of the arterial wall, for example, keratan sulphate reported to be present by Buddecke (1960), has not been found by other workers (Antonopoulos, Gardell and Hamnström, 1965; Meyer, Davidson, Linker and Hoffman, 1956). Again Murata and Kirk (1962) report a sialic acid content for human aorta of 0.178 g percent of the wet weight. These authors have compared this to a value of 7.8 percent quoted by Buddecke (1962) which is presumably the value obtained on a dry weight basis. Regrettably, but perhaps understandably, the use of a wide variety of tissue samples e.g. intima (Radhakrishnamurthy et al., 1964; Klynstra, Böttcher, van Melsen and van de Laan, 1967), intima plus media (Barnes, 1965; Muir, 1965; Dunstone, 1967), media (Klynstra et al., 1967), adventitia (Murata and Kirk, 1962) and the full thickness of the tissue (Antonopoulos et al., 1965; Buddecke et al., 1963), and an even more variable array of chemical, histological and histochemical techniques have contributed to the present situation.

I:3. THE PROTEINPOLYSACCHARIDES OF CONNECTIVE TISSUE.

I:3:I. NOMENCLATURE.

The systematic nomenclature and classification of many polysaccharides presents considerable problems and it has become usual to group the polysaccharides into broad groups according to their sources and biological functions, e.g. the amino sugar-containing polysaccharides found in connective tissue.

Meyer (1938) adopted the term mucopolysaccharide for these substances and their salts, the prefix 'muco' denoting a relationship between this group of polysaccharides and mucous, the physiological term for a viscous secretion. Subsequently, the term mucopolysaccharide has been used to denote severally, a polysaccharide containing more than 4 percent hexosamine (Meyer, 1945), a polysaccharide containing hexosamine in any proportion (Fishman, 1951), protein-containing polysaccharide substances of high protein or peptide content and lipid-containing polysaccharides (Bettelheim-Jevons, 1958; Kent and Whitehouse, 1955).

More recently there has been a trend for the term mucopolysaccharide to be applied only to those heteroglycans which contain residues of both uronic

acid and hexosamine (Pigman and Goepp, 1948; Jeanloz, 1956). Attention was thereby confined to a group of substances including hyaluronic acid, chondroitin sulphate, heparan sulphate and heparin which are conveniently called acid mucopolysaccharides.

However, ambiguity has arisen in relation to a number of carbohydrate polymers that are closely associated with these acid mucopolysaccharides in the living tissue but which cannot be strictly classified with them since they do not contain uronic acid residues, for example keratan sulphate which contains galactose but no uronic acid (Hirano, Hoffman and Meyer, 1961).

This definition has failed also to distinguish between proteinpolysaccharides which contain large or small amounts of protein; it does not distinguish between the mucopolysaccharides which contain uronic acid and/or sulphate and those which do not and finally some protein-containing polysaccharides which are similar in many respects were excluded because of the presence of only very small amounts of hexosamine (Stacey, 1943, 1946).

A valuable attempt at a systematic nomenclature for these structurally complex polysaccharides

has been made by Jeanloz (1960). However, due to the cumbersome nature of the names involved, i.e. glycosaminoglycuronoglycan, (the systematic name proposed by Jeanloz (1960) for the uronic acid-containing mucopolysaccharides), their general use has been limited. In general, the mucopolysaccharides have retained their trivial names which were allotted at the time of their discovery.

Generally, acid mucopolysaccharides when present in connective tissue are associated with protein or peptide residues (Shatton and Schubert, 1954; Partridge and Davis, 1958). However, since little is known about the structure of these substances, little in the way of classification or nomenclature can be assigned to them. When the presence of these materials was first noticed, terms such as "chondromucoprotein" were coined (Malawista and Schubert, 1958). Later it was considered better to call these substances by non-committal terms such as "proteinpolysaccharide complex" or more explicitly "a hyaluronic acid protein complex" (Jeanloz, 1960), "a chondroitin sulphate complex" (Muir, 1958). More recently Tsiganos and Muir (1967) have referred to these compounds collectively as proteoglycans.

Schubert (1961) challenges the use of the term 'complex' because it implies the opposite to what is generally accepted as the chemical definition i.e. as in a co-ordination compound or complex. He suggests that if a word does not have a definable meaning it should not be used; further, since a lipid-protein association is referred to simply as a lipoprotein there is no reason why a carbohydrate-protein association should not be referred to simply as a proteinpoly-saccharide or a polysaccharideprotein.

The classifications proposed by Meyer (1953) and Jeanloz (1960) are essentially similar with regard to actual classifications but differ with respect to the nomenclature. However, since these proposals were put forward the situation has been further compli-cated; it is now evident that these materials are far more complex than was originally visualized. For example, the proteinpolysaccharide extracted from nasal cartilage appears to have more than one protein fraction associated with it (Partridge, Whiting and Davis, 1965); further, the carbohydrate residue itself contains more than one polysaccharide component (Gregory and Rodén, 1961). Finally, where in some cases it has been possible to further fractionate some of these protein-

polysaccharide substances, much ambiguity has resulted by alloting the fractionated species laboratory code names only, without attempting to identify these with materials of known composition.

In view of this situation there can be no dissent for use of the term 'complex' if used in a non-chemical sense (i.e. meaning composite), when referring to a mixture of proteinpolysaccharide compounds that to all appearances are closely related in the living tissue. The term proteinpolysaccharide refers only to a single carbohydrate protein substance, which behaves as a single entity, regardless of the amount of protein associated with it, i.e. it is a general term assigned to substances previously classified as glycoproteins or as acid mucopolysaccharide protein complexes.

The classification and nomenclature to be used in this thesis (excluding the General Introduction) are itemized below. An attempt has been made to refer to material isolated from connective tissue as 'protein-polysaccharide material' or simply 'proteinpolysaccharide'. However, if more than one proteinpolysaccharide is known to be present in a given preparation, then the term proteinpolysaccharide complex will be used. When possible the nomenclature for the polysaccharide com-

ponents recommended by Jeanloz (1960) has been used. In such cases the specific polysaccharide name precedes the word, protein.

Class I. Glycosaminoglycuronoglycan Proteins

Group A. When only one type of glycosaminoglycuronoglycan is associated with the protein, the material will be referred to specifically, i.e. a chondroitin sulphate protein, a hyaluronic acid protein.

Group B. When more than one type of polysaccharide of this class is associated with a single protein material it will be referred to by the class name, i.e. a single macromolecule containing both chondroitin sulphate and keratan sulphate in association with protein, such as that found in nasal cartilage will be referred to as a glycosaminoglycuronoglycan protein.

Class II. Glycosaminoglycan Proteins

This class comprises those proteinpolysaccharides which do not contain uronic acid.

Group A. The materials in this group are similar to those in Class I, group A, in that they are predominantly carbohydrate and were previously grouped among the 'mucopolysaccharides'.

Keratan sulphate protein is a member of this group.

Group B. This group comprises those proteinpolysaccharides which contain protein as the major constituent. Previously, the components of this group have been called glycoproteins; the latter term will be used to refer to the class II, group B compounds, throughout this thesis.

I:3:2. INTRODUCTION TO PROTEINPOLYSACCHARIDE STRUCTURE.

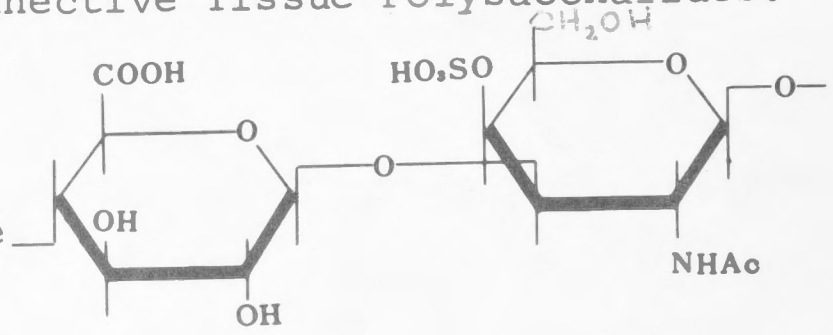
Early studies of these materials invariably involved treatment of the connective tissue under relatively vigorous conditions, such as prolonged digestion with proteolytic enzymes, extraction with strong salt solutions or dilute alkali. The resultant extracts yielded, by several methods of purification, varying amounts of one or more of a group of carbohydrate polymers. In defining the purity of such materials early workers attached much significance to the absence of amino acids and polypeptide residues from the polysaccharide preparation and when demonstrated, such 'contamination' was frequently attributed to the inefficiency of the purification procedures (see for example Mathews, 1955).

Structural studies on these compounds reveal

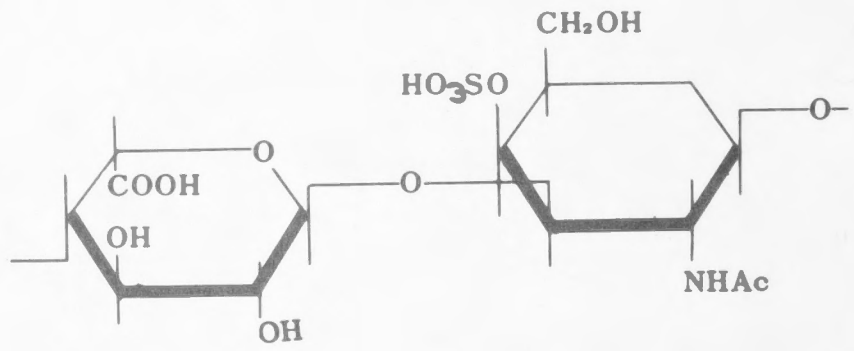
Fig.1:1a. The Chemical Structure of the various repeating Units in Connective Tissue Polysaccharides.

Repeating unit of:

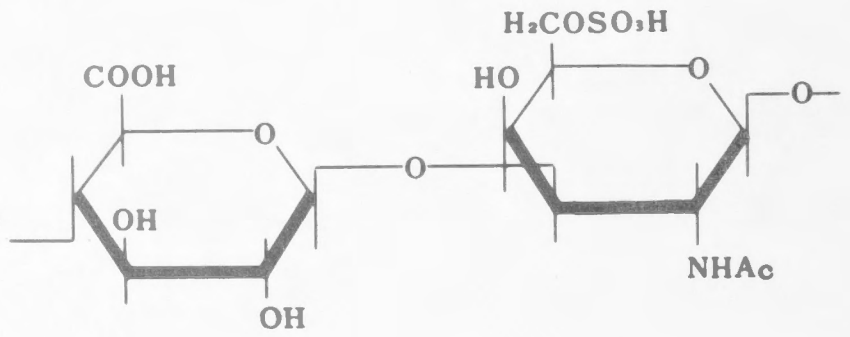
Chondroitin 4-sulphate



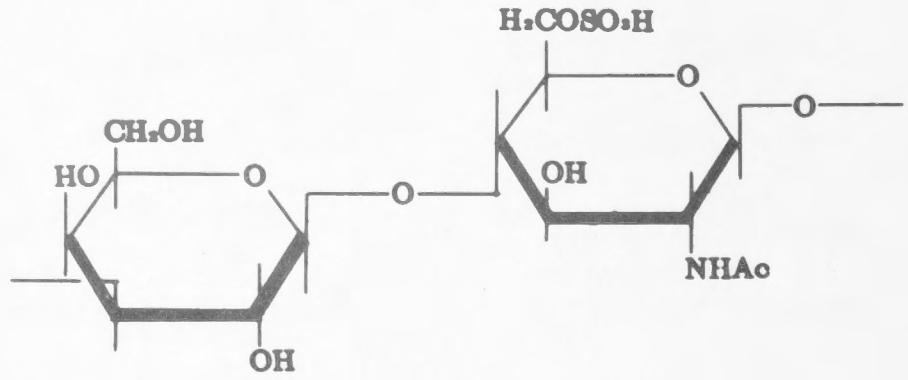
Dermatan sulphate



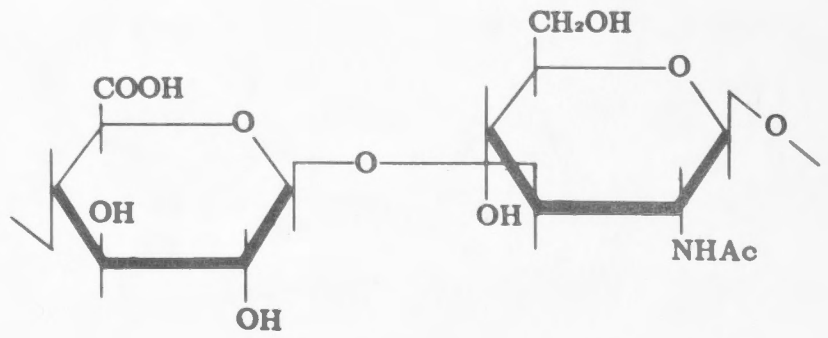
Chondroitin 6-sulphate



Keratan sulphate



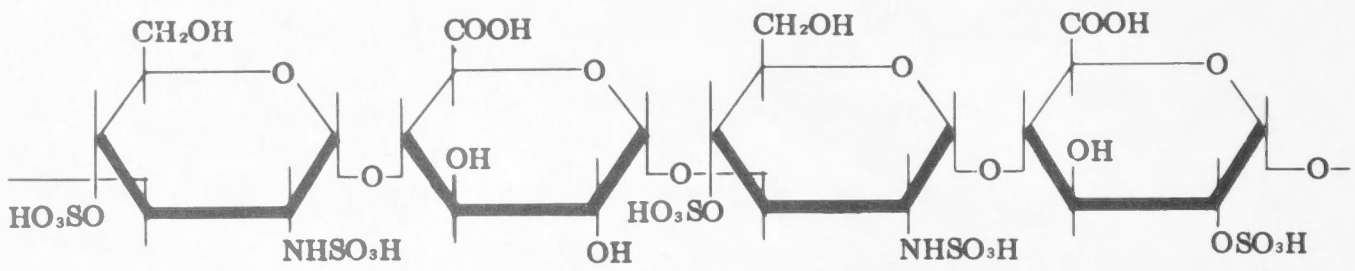
Hyaluronic acid



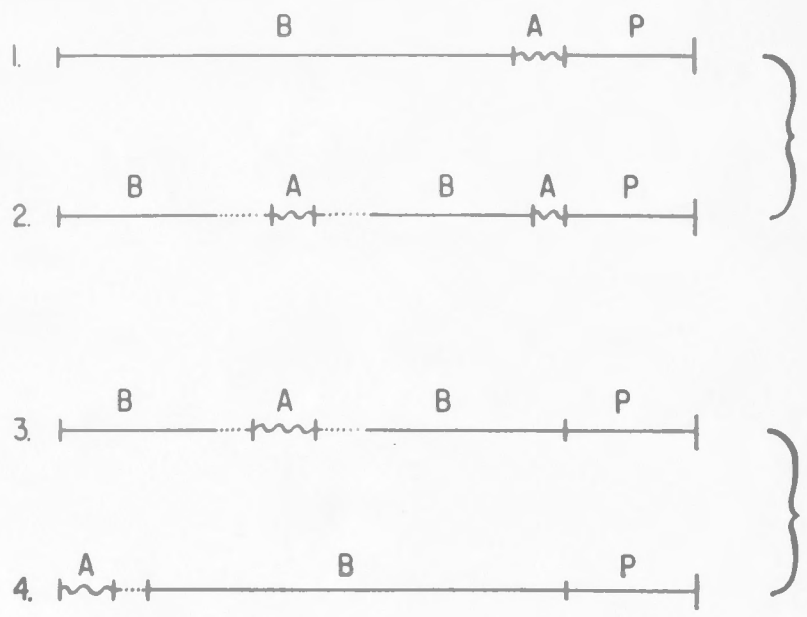
a pattern of similar molecular organization (Jeanloz, 1963) i.e. they are composed of linear monosaccharide chains of relatively high molecular weight. Hexosamine residues, (D-glucosamine or D-galactosamine), alternate regularly in the polymer chains with other monosaccharides, which may be, D-glucuronic acid, L-iduronic acid or a hexose such as galactose. The chemical structures of the various repeating units making up these polymers are illustrated in fig. I:I a and b. The structure for heparan sulphate is not shown because as yet it is imperfectly characterized. Linker and Sampson (1960) have suggested that the polymer is a hybrid molecule consisting of repeating units of D-glucosamine di-sulphate-D-glucuronic acid and N-acetyl D-glucosamine - D-glucuronic acid. A more recent view of the structure of heparan sulphate has been given by Knecht, Cifonelli and Dorfman (1967). Fransson and Rodén (1967a,b) have presented evidence which shows that dermatan sulphate contains glucuronic acid in addition to iduronic acid; a schematic model of some possible hybrid structures of dermatan sulphate is illustrated in fig I:Ib.

Table I:I gives a brief summary of the distribution of these polysaccharides in some connective tissues. This summary is not exhaustive, but is simply

Fig. 1:1b. The structure of various repeating units in connective tissue polysaccharides.



Repeating unit - heparin



Schematic models of some possible hybrid structures of dermatan sulphate. A, sections containing D-glucuronic acid; B, sections containing L-iduronic acid; P, polysaccharide bound peptide (Fransson and Roden, 1967b).

used to illustrate the complexity of the distribution of the polysaccharides in some tissues compared with others.

These polysaccharides are also polyanions; the negative charge arises either from the carboxyl group of the hexuronic acid or from the presence of ester bound sulphate, or both. As the carboxyl and the sulphate groups are fully ionized at physiological pH, the resultant highly negative character is likely to have considerable bearing on the functions of these polymers in the native state.

The pure polysaccharides, required for structural studies, do not exist as such in the native tissue. Application of milder methods of extraction, such as physical disintegration of the tissues by high speed homogenization (Shatton and Schubert, 1954; Gerber et al., 1960), yields a carbohydrate polymer firmly linked to polypeptide (Partridge and Davis, 1958).

In recent years, by far the most exhaustive studies of the proteinpolysaccharides from the ground substance of connective tissue have been performed with mammalian hyaline cartilage. This tissue is readily available in comparatively large amounts, and has, in comparison with other tissues, a relatively simple carbohydrate composition. For this reason it has been

Table 1:1. Distribution of Connective Tissue Polysaccharides

TISSUE	HA	C-4-S	C-6-S	DS	KS	HS	H	REF.
Aorta	BH	BH	BH	BHP	(?)H	BH	B	1.
Skin	P	P	P	BPRS				1,6,7.
Tendon	B	R	BP	P				1,8.
Cartilage		BH	BHS		BS			1,2,3,4,7,8.
Bone	H	B			B			1.
Heart Valve	BP		BP	BP				1,5,11.
Ligamentum Nuchae		B		P				1.
Synovial Fluid	B	B						2,10.
Cornea		B			B			1.
Intervertebral disc			B		B			9.
Umbilical cord	BH							1.

Abbreviations: HA, hyaluronic acid; C-4-S, chondroitin 4-sulphate; C-6-S, chondroitin 6-sulphate; DS, dermatan sulphate; KS, keratan sulphate; HS, heparan sulphate; H, heparin.
B, bovine; H, human; P, porcine; R, rat; S, shark.

REF. References:
1. Meyer, Davidson, Linker and Hoffman (1956)
2. Muir (1958)
3. Anderson (1962)
4. Mathews (1962)
5. Moretti and Whitehouse (1963)
6. Seno and Meyer (1963)
7. Meyer (1964)
8. Lloyd (1965)
9. Lowther and Baxter (1966)
10. Silpananta, Dunstone and Ogston (1967)
11. Lowther, Toole and Meyer (1967).

extensively used for the characterization of the proteinpolysaccharide macromolecule.

Aorta, on the other hand, has an extremely complex array of proteinpolysaccharides; it is thus not the most suitable tissue, in the light of present knowledge, to use as a convenient model in which to visualize the macromolecular constitution of the proteinpolysaccharides. Nevertheless, aorta is a useful system to employ when investigating techniques for the separation and isolation of several proteinpolysaccharides or the polysaccharides only, within a single system (Antonopoulos et al., 1965; Thunell, Antonopoulos and Gardell, 1967; Dunstone, 1967).

I:3:3. THE PROTEINPOLYSACCHARIDE OF BOVINE NASAL CARTILAGE.

The proteinpolysaccharide isolated from bovine nasal cartilage represents 40-50 percent of the dried cartilage weight (Malawista and Schubert, 1958; Rotstein, Gordon and Schubert, 1958; Pal, Doganges and Schubert, 1966). By means of high speed homogenization it is possible to extract up to 80 percent of the total carbohydrate material in the tissue (Malawista and Schubert, 1958). The isolated product contains 15-25 percent protein and 75-85 percent carbohydrate

(Gerber et al., 1960). Further purification of this crude product by ultracentrifugation, yields a protein-polysaccharide material called PPL by the authors and represents about 40 percent by weight of the dried cartilage (Gerber et al., 1960).

The carbohydrate moiety is composed of chondroitin 4-sulphate, keratan sulphate and smaller amounts of the isomeric chondroitin 6-sulphate (Buddecke et al., 1963; Partridge, 1966b).

I:3:3:I. The Proteinpolysaccharide as a Macromolecule.

Morner (1889) and Schmiedeberg (1891) isolated carbohydrate protein products from alkaline extracts of cartilage to which they gave such names as chondromucoid and peptochondrin. Morner (1889) held that the chondroitin sulphate occurred in the tissues as an alkali metal salt. In later work, Meyer, Palmer and Smyth (1937) considered that the chondroitin sulphate occurred in the tissues in salt-like complexes which were present as structurally organized elements in sheet or fibre form. They were able to show that in the presence of acetic acid, true salts were formed in stoichiometric proportions, by the union of the basic groups of the protein and the acid groups of the polysaccharide. In the salts studied, chondroitin

sulphate was found to react with the protein as a dibasic acid; both the sulphate groups and the carboxyl group of the uronic acid taking part in the salt formation.

Partridge (1948) made a preliminary study of the intact tissue, by examining the breakdown products, resulting after successive mild degradation of one or other of the tissue components. Mucoid material was extracted into aqueous solution, after heat treatment to 'shrink' the collagen. Materials extracted in this way, when examined electrophoretically, were found to behave as equilibrium mixtures of protein, carbohydrate and also as a complex of the two. Partridge (1948) suggested that the complex was due to the strongly acidic groups of the carbohydrate being held in combination with some of the basic groups of the protein. However, since it was not possible to liberate the mucoid without modifying the collagen, the results could not be overlooked as artifacts.

Einbinder and Schubert (1950) and Shatton and Schubert (1954), while investigating mild methods for the extraction of chondroitin sulphate from cartilage, obtained the first real evidence that the

chondroitin sulphate did in fact exist in cartilage as a proteinpolysaccharide compound. The product, extracted with water from acetone-dried cartilage, was found to contain both polysaccharide and protein. Determination of the hydroxyproline content gave values always less than 0.5 percent; the protein fraction could not therefore be collagen. Further this product gave no precipitation reaction with any of the common protein precipitating reagents and analyses of the salts of the mucoprotein with potassium, barium and a complex cobalt cation were observed to be remarkably constant in composition. Shatton and Schubert (1954) offered this as evidence that the product behaved as a compound and, in keeping with the classification of Meyer (1945), termed it a mucoprotein. Later, Malawista and Schubert (1958), referred to this compound as chondromucoprotein, while Muir (1958) referred to similar material as a chondroitin sulphate complex because no evidence was found for the presence of carbohydrate other than chondroitin sulphate. However, Muir (1958) was of the opinion that the protein could not be held by electrostatic forces because her complex had been extracted with neutral $CaCl_2$ and the extract subsequently saturated with ammonium sulphate,

processes which are known to disrupt electrostatic bonds. Further, the complex migrated as a single boundary during electrophoresis and did not dissociate even when the ionic strength was increased. Warner and Schubert (1958) reported similar findings.

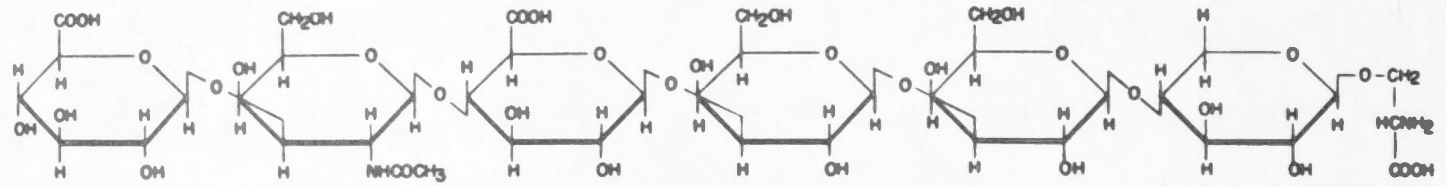
Partridge and Davis (1958) confirmed by two independent experiments that part, at least, of the chondroitin sulphate in cartilage was in firm chemical combination with a non-collagenous protein. These authors extracted the mucoprotein by the method of Einbinder and Schubert (1950) and removed collagenous protein by means of ion-exchange resins; zonal electrophoresis carried out on the undegraded material and on material degraded by acid and alkaline hydrolysis, showed that the undegraded mucoprotein moved as a single boundary containing both protein and polysaccharide. The degraded material on the other hand showed two boundaries, one of chondroitin sulphate and one of protein. Since repeated treatment with ion-exchange resins failed to reduce the protein content of the undegraded material to less than 10 percent these authors concluded that the non-collagenous protein must therefore be firmly bound, possibly in chemical combination with the polysaccharide.

I:3:3:2. Nature of the Linkage between the Carbohydrate and the Protein Moieties.

Three types of protein to polysaccharide linkages have been described: (I) a glycosidic linkage between the hydroxyl group of serine and xylose (Muir, 1958; Rodén and Smith, 1966), (2) a similar linkage through threonine (Anderson, Seno, Sampson, Riley, Hoffman and Meyer, 1964) and (3) a linkage between aspartic acid and the carbohydrate (Seno, Meyer, Anderson and Hoffman, 1965). Of these, the first has been the most extensively studied in nasal cartilage protein-polysaccharides.

Muir (1958), obtained the first evidence that serine was involved in the linkage. Preferential destruction of the serine by treatment with alkali (Anderson, Hoffman and Meyer, 1963) provided further evidence that the hydroxyl group of the serine was involved. No evidence was found to suggest that any other amino acid was involved (Anderson, Hoffman and Meyer, 1965).

Investigating the sequence in the carbohydrate residues obtained after enzymic degradation of the nasal cartilage, Rodén, Gregory and Laurent (1964) found that galactose and xylose were implicated. These



-GlcUA-(1→3)-GalNAc-(1→4)-GlcUA-(1→3)-Gal-(1→3)-Gal-(1→4)-Xyl-O-Ser

Fig. 1:2. Nature of the linkage between the carbohydrate and the protein moieties of bovine nasal cartilage (Roden and Smith, 1966).

workers were able to show that uronic acid and galactosamine were present in a ratio of 2:1, while galactose, glucuronic acid and xylose were present in the ratio 2:2:I (Rodén, Gregory and Laurent, 1963; Gregory, Laurent and Rodén, 1964). Rodén (1965) reasons that such results demand that the alternating disaccharide units be maintained in the linkage region, however the 2:I ratio of glucuronic acid to galactosamine required a residual trisaccharide to be present, i.e. GlcUA - GalNAc-GlcUA. This fact was substantiated by the isolation of a disaccharide unit of glucuronic acid linked by a β glucuronidic bond to galactose, the latter being at the reducing end (Rodén, 1964). Later, Lindahl and Rodén (1964, 1966) and Rodén and Lindahl (1965) were able to characterize several terminal oligosaccharides, enabling Rodén and Smith (1966) to propose a structure for the carbohydrate moiety in the linkage region. This structure is illustrated in fig I:2.

I:3:3:3. The Molecule Size, Particle Shape and Heterogeniety of Nasal Cartilage Proteinpolysaccharide.

(i) Molecular Size and Particle Shape.

Mathews (1955) and Webber and Bayley (1956)

reported values of 1.0×10^6 for the molecular weight of the proteinpolysaccharide isolated from bovine nasal cartilage, as determined by sedimentation and viscosity. Bernardi (1957), on the other hand, found values of 2.0×10^6 and 0.48×10^6, respectively for the weight-average and number-average molecular weights, indicating considerable polydispersity.

Webber and Bayley (1956) have described the molecule as containing approximately 20 chondroitin sulphate chains, each of molecular weight 3 to 4×10^6. However, Muir (1956) considered that the chondroitin sulphate chains which had molecular weights of this order, represented aggregates of smaller polysaccharide molecules, linked together by small amounts of peptide or protein. Mathews (1956) was of a similar opinion. He considered that the higher values reported in some instances, e.g. 2.6×10^5 (Blix and Snellman, 1945) were due to contamination of the preparation with proteinpolysaccharide material.

The polysaccharide residue obtained by Webber and Bayley (1956) after tryptic digestion of the proteinpolysaccharide had a number-average molecular weight of 3×10^4 and a weight-average molecular weight of 14.5×10^4. The product was accordingly regarded

as being widely polydisperse. A different conclusion was reached by Mathews (1955, 1956), who isolated chondroitin sulphate protein material from hyaline cartilage and after treating with proteolytic enzymes claimed that this product was monodisperse with a molecular weight of about 5×10^4.

Later, Mathews and Lozaityte (1958), from light scattering and viscosity data, proposed that the basic molecular unit of the chondroitin sulphate protein (as determined in a phosphate buffer of ionic strength 0.4), was a rod of length 3700A having a molecular weight of 4.0×10^6. To account for the linear distribution of matter, these authors proposed that the protein moiety formed a core running the length of the rod and along which were distributed 62 chondroitin sulphate chains, each of molecular weight 5×10^4. Evidence was also presented, which suggested that aggregations of much higher molecular weight may be formed, by lateral and end-to-end association of the basic units. Further, these authors were of the opinion that additional protein material may also be involved within the macromolecular structure.

These results conflicted with those of Webber and Bayley (1956) and Bernardi (1957, 1959), who

favoured a coiled filament, consisting of linear chondroitin sulphate chains bridged by polypeptide chains in an end-to-end arrangement. However, investigation of the kinetics of degradation of such proteinpolysaccharide preparations indicated that the model proposed by Mathews and Lozaityte (1958) was the more probable (Cessi and Bernardi, 1965).

Partridge, Davis and Adair (1961), obtained from acetone-dried and from fresh cartilage, a crude proteinpolysaccharide preparation containing approximately equal amounts of uronic acid and galactosamine (20 percent w/w) as well as a small amount of glucosamine (3.4 percent w/w). Determination of the chain weight of such preparations and also of material degraded by alkali and by enzymes, indicated that each molecule of proteinpolysaccharide contained 23 chains of polysaccharide (each of molecular weight 2.8×10^4) attached to a protein core. The protein core (containing considerable amounts of neutral sugars and hexosamine), was estimated to have a molecular weight of 12.0×10^4. These workers proposed a model for the complex which is similar to that described by Mathews and Lozaityte (1958), but with the additional feature that a second polysaccharide polymer is linked covalently, in a

terminal position, to the protein core. The second polysaccharide was considered to be keratan sulphate (Meyer, Linker, Davidson and Weissmann, 1953; Partridge and Elsden, 1961; Gregory and Rodén, 1961). The molecular weight of the whole unit was estimated as 0.75×10^6 (protein core, 12.0×10^4, plus 23 polysaccharide chains, each of molecular weight 2.8×10^4). All higher molecular weight material encountered was considered to be aggregates.

Buddecke et al. (1963) and Luscombe and Phelps (1967a, b), were basically in agreement with the protein core model. These workers have isolated proteinpolysaccharide material from bovine nasal cartilage by similar methods.

Buddecke et al. (1963) characterized their product as having a molecular weight of 0.47×10^6, containing 15-20 percent protein and having 20 to 30 chondroitin sulphate chains each of molecular weight 2×10^4 attached to a single protein core. Luscombe and Phelps (1967a, b), on the other hand, obtained a significantly higher molecular weight (3.2×10^6) but considered the molecule (containing 20 percent protein) to be composed of four or five protein cores associated with 100 chondroitin sulphate chains each made up

Table 1:2. Characteristics of the Proteinpolysaccharide Macromolecule isolated from Bovine Nasal Cartilage. (Figures given are the average of those quoted by the various authors.)

PP	CHONDROITIN SULPHATE CHAIN			PROTEIN		REF.
10^{-6} x MW	10^{-4} x MW	No. Chains	No. disacc. residues/chair	percent	10^{-4} x MW	
1.00	4.0	20	(80)	15	(15)	1.
4.00	5.0	62	(100)	25	(90)	2.
0.75	2.8	23	(60)	-	12	3.
0.47	2.0	25	(40)	20	(9)	4.
0.24	2.8	9	(60)	7	2	5.
0.75	1.8	40	(40)	(3)	25	6,7.
3.20	2.2	100	40	20	-	8,9.
(0.60)	2.2	23	40	-	12	8,9.
3.00	13.0	28	(240)	15	(40)	10.

Values quoted in brackets have been calculated from the results presented by the various authors.

Abbreviations: MW, molecular weight; PP, proteinpolysaccharide; disacc, disaccharide units.

REF. References:
1. Webber and Bayley (1956).
2. Mathews and Lozaityte (1958).
3. Partridge, Davis and Adair (1961).
4. Buddecke, Kroz and Lanka (1963).
5. Partridge (1966b).
6. Meyer (1966a).
7. Meyer (1966b).
8. Luscombe and Phelps (1967a).
9. Luscombe and Phelps (1967b).
10. Buddecke, Kroz and Tittor (1967).

of approximately 40 disaccharide units.

The findings of each of these groups of workers are included in table I:2. If it is assumed that the molecular weight found by Buddecke et al. (1963) is correct, then the material described by Luscombe and Phelps (1967a, b), having a molecular weight of 3.2×10^6 and containing 4 to 5 protein cores, could not represent the basic molecular unit. However, by considering the basic unit as containing a single core plus its associated chondroitin sulphate chains (i.e. about 20 chains each of molecular weight 2.2×10^4) then the basic unit would have a molecular weight of $0.5 - 0.6 \times 10^6$, in reasonable agreement with the results of Buddecke et al. (1963).

However, these findings are not in agreement with the more recent investigations of Buddecke, Kroz and Tittor (1967), who describe the proteinpolysaccharide as having a molecular weight of 3.0×10^6 but containing only 24 to 32 chondroitin sulphate chains bound to the protein. The protein moiety represents only 12-18 percent of this weight; this implies that the chondroitin sulphate chains each have a molecular weight of the order of 13.0×10^4 a value considerably higher than those reported by other workers, which are usually in the range

2.0 - 5.0 x 10^4 (see table I:2).

Meyer (1966a, b) claimed that chondroitin sulphate chains isolated by enzymic digestion and reported as having molecular weight of 2 - 5 x 10^4, were aggregated forms and that the true molecular weights, (1.5 - 1.8 x 10^4) were only obtained after alkali treatment of the proteinpolysaccharide material. Further, Anderson et al. (1963) have examined the amino acid profiles of material degraded in this way and have concluded that 40 serine residues (and thus 40 polysaccharide chains) are present in each molecule.

Meyer (1966a, b) has reasoned that, if the molecular weight of the proteinpolysaccharide is assumed to be 7.5 x 10^5 (Partridge et al., 1961), the carbohydrate moiety consisting of 40 chains each of 1.8 x 10^4 molecular weight will have a total molecular weight of 7.2 x 10^5, leaving a polypeptide backbone of molecular weight of 2 - 3 x 10^4. Further, the average molecular weight of a single amino acid is of the order of 100, so the protein core will contain 200 - 300 amino acid residues. Assuming that the carbohydrate chains are distributed evenly along the protein backbone, there would be one carbohydrate chain every 5-7 amino acid residues or one chain about every hexapeptide. Meyer

(1966a) has concluded that since the peptido-polysaccharides produced by many proteolytic enzymes have molecular weights of 3 - 5 x 10^4 they are in fact doublets. To explain this, Meyer (1966a) has speculated that the polypeptide backbone is a double coil with the polysaccharide side chains sticking out in the shape of a bottle brush.

A summary of some molecular weights and particle dimensions of the proteinpolysaccharides and related fractions are shown in table I:2. Some estimations inferred from the results of the various groups of workers are also included.

The above information suggests, that before a satisfactory evaluation of the molecular weight and particle shape can be made, a definition as to what constitutes a single macromolecular species is essential; further, it is important to consider whether the molecular species to be studied is an artifact resulting from the method of extraction or whether it exists as such in the native tissue.

(ii). Heterogeneity

It is now generally agreed that the material isolated from cartilage and previously described as homogeneous (Gerber et al., 1960), is in fact, not homogeneous. Pal et al. (1966) found that such preparations were chemically heterogeneous and ultracentrifugal heterogeneity could be

observed in solutions of high ionic strength, provided the solutions were sufficiently dilute. A similar finding was also reported by Webber and Bayley (1956) but has apparently passed unnoticed by many workers.

On the other hand, Buddecke et al. (1967) have inferred, from chemical analysis, that there exists in bovine nasal cartilage, several types of proteinpolysaccharide which are chemically similar, but which are very polydisperse with regard to molecular weight. Mashburn, Hoffman, Anderson and Meyer (1965) considered that even the mildest methods of extraction yielded material of sufficient electrophoretic heterogeneity to prevent the definition of a discrete protein-polysaccharide.

The variable protein content of protein-polysaccharides, obtained in different preparations, led Mathews and Lozaityte (1958) to suggest that extraneous protein might participate in the formation of various aggregated forms. This could explain the observed heterogeneity.

Partridge and Elsden (1961) observed that protein material isolated from preparations by alkali degradation was electrophoretically heterogeneous.

Evaluation of the sialic acid content of

human cartilage (Anderson, 1961, 1962) showed that it was extremely difficult to obtain fractions free of this acid, which did not appear to be involved in the linkage of the carbohydrate and protein moieties. Interpreting these findings, Anderson (1962) suggested that the sialic acid could be a constituent of a glycoprotein or a family of glycoproteins, or the protein core may itself be a glycoprotein.

Fitton Jackson (1965) examined a preparation similar to that described by Gerber et al. (1960) (i.e. PPL) in the electron microscope and distinguished masses of subunits 45-55 A across frequently in groups of 5 or 6 arranged around a central core of mean width 165 A. These observations suggested the presence of a second protein, globular in nature, distinct from the protein of the polypeptide core. Partridge and coworkers, (1965, 1966b), seem to have successfully isolated such a protein. The proteinpolysaccharide, from which the globular protein had been removed, was found to have a molecular weight of 2.4×10^5; considerably smaller than those reported previously.

Buddecke and coworkers (1963, 1967), while recognizing that many of the observed differences between the various preparations could be explained by

the differing processes of isolation and purification that had been used, were of the opinion that the observed differences in macromolecular properties were the result of differences in ionic environment. Their light scattering data suggested that the macromolecules were rod shaped (approximately 3,000 A in length) when examined in distilled water. The molecular weight of several preparations differed by a factor of 3-4 while the corresponding radius of gyration differed by only a factor of 1.5; it was inferred therefore that some of these preparations must include aggregates or micelle formations. The marked influence of the electrolyte concentration is illustrated in the following example: in 0.2 M NaCl, molecular weights of up to 60×10^6 were observed whereas the same preparation in water had a molecular weight of only 3.7×10^6.

These authors visualized the proteinpoly-saccharide macromolecule as a system of macro-ions which has a tendency to alter volume and aggregate as the electrolyte conditions are changed. Thus, in distilled water, individual carbohydrate chains become expanded and the chains separate from each other due to electro-static repulsion of like charges; under these conditions, the molecules take up a rod-like configuration. When

the ionic strength is increased, anionic charges are screened off by counterions and the electrostatic repulsion is largely removed; the rod-shaped molecules then roll up into statistical coils, which tend to aggregate, forming molecules with molecular weights of the order of 8×10^7 and having more nearly spherical configurations.

Such observations also reflect the findings of Partridge (1966b) and Luscombe and Phelps (1967a, b) who visualize the macromolecule as containing polysaccharide chains, (randomly coiled around the protein core) and tending to form spherical configurations with the more hydrophobic material directed toward the centre. Further, because of the polyelectrolyte nature of the proteinpolysaccharide, it is possible that the separate cores and associating chains will aggregate in the presence of basic protein or glycoprotein.

It would appear that the proteinpolysaccharide from bovine nasal cartilage, although relatively simple in chemical composition is very complex structurally. As yet there is no clear conception as to what constitutes the basic macromolecular unit, or indeed if we are justified in endeavouring to try and isolate such a unit. It would seem that the protein-

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polysaccharide is composed of a protein core along which are distributed at regular intervals polysaccharide chains (Mathews and Lozaityte, 1958; Partridge and coworkers, 1961, 1965, 1966b; Buddecke and coworkers, 1963, 1967; Luscombe and Phelps, 1967a, b). The exact number of the carbohydrate chains is difficult to ascertain. Values between 20 and 30 are the most consistently quoted. However, if the proteinpolysaccharide does include a protein distinct from the protein of the protein core, it would be expected that the molecular unit described by Partridge (1966b) containing only 9 chains per molecule would represent the more real image.

In general, studies of the proteinpolysaccharide from bovine nasal cartilage have been concerned with either characterizing the carbohydrate moiety or with characterizing the proteinpolysaccharide as a macromolecule. However, in the light of more recent findings it would appear that before a definite understanding of the proteinpolysaccharide as a macromolecule can be obtained, more emphasis must be placed on obtaining a clear conception of the protein moiety, including also, any extraneous protein or glycoprotein that may be present and may or may not be involved in maintaining the macromolecular integrity. Further, in order to

obtain a better understanding of possible functions such a secondary protein species might have in the macromolecular structure, it is imperative that it be isolated from the proteinpolysaccharide proper by as mild a means as possible so that examinations might be made on these substances both as isolated species and in the presence of each other.

I:3:4. THE PROTEINPOLYSACCHARIDES OF AORTA

Most of the commonly occurring glycosaminoglycuronoglycans have been identified in aqueous extracts of aorta, i.e. hyaluronic acid (Meyer et al., 1956; Antonopoulos et al., 1965); chondroitin 4-sulphate (Buddecke et al., 1963); chondroitin 6-sulphate Meyer et al., 1956; Buddecke et al., 1963; Antonopoulos et al., 1965); dermatan sulphate (Meyer et al., 1956; Antonopoulos et al., 1965); heparan sulphate (Jacobs and Muir, 1963; Muir, 1965; Antonopoulos et al., 1965) and heparin (Meyer et al., 1956). Keratan sulphate has been reported in one isolated instance, (Buddecke et al., 1963) however, Antonopoulos et al. (1965) and Thunell et al. (1967) have been unable to demonstrate its presence. Recently it has been established that a family of materials resembling glycoproteins are also present in aorta (Berenson, 1961; Fishkin, Berenson and Kantrow, 1961; Berenson

and Fishkin, 1962; Seng, Suzuki, Werber and Voigt, 1965; Kind, Seng, Werber and Voigt, 1964).

The proteinpolysaccharide material from arterial walls accounts for approximately 1.3 percent of the dried tissue weight (Buddecke, 1960) while the glycosaminoglycuronoglycans represent about one third of this value. The composition of the glycosaminoglycuronoglycans is typically as follows: chondroitin sulphate (4- and 6- sulphate) and dermatan sulphate 40-50 percent, hyaluronic acid 15 percent and heparan sulphate 10 percent of the total glycosaminoglycuronoglycan content (Buddecke, 1962; Meyer, 1964). The total amount of glycosaminoglycuronoglycan material in human aorta does not vary much with age, however the relative proportions change markedly with age and/or with disease. Generally the proportion of hyaluronic acid is found to decrease as age and disease progress (Buddecke, 1962; Kaplan and Meyer, 1960; Milch, 1965; Klynstra et al., 1967) while the proportions of dermatan sulphate and heparan sulphate are found to increase (Meyer, 1964). Further, Kaplan and Meyer (1960) have shown that chondroitin 6-sulphate decreases with age, however Buddecke (1962) has reported increased amounts. Chondroitin 6-sulphate also appears in

increased amounts in diseased tissues (Buddecke, 1962; Klynstra et al., 1967).

Variation in the distribution of these polysaccharides has also been detected across the width of the arterial wall (Berenson, 1961; Klynstra et al., 1967) and throughout the length of the artery (Manley and Hawksworth, 1965). These latter authors have found that in general the ratio of hyaluronic acid to chondroitin sulphate increased as one proceeded from the aorta to the peripheral arteries, or in other words, the glycosaminoglycuronoglycan pattern gets 'younger' as the arterial tree is descended. Other workers have reported genetic variations in the proteinpolysaccharide distribution. Nakamura, Isihara, Sata and Yabuta (1966) have observed that the glycosaminoglycuronoglycan content in the aortas of Japanese persons is less than in persons of Caucasian descent. Similarly Berenson, Radhakrishnamurthy, Fishkin, Dessauer and Arqeumbourg (1966) have found variations in the glycoprotein pattern between American Negroes and Caucasians, between the different sexes and in persons of different blood groups.

I:3:4:I. The Glycosaminoglycuronoglycan Proteins of Aorta

To date only two proteinpolysaccharides from

the glycosaminoglycuronoglycan protein complex in aorta, namely, those containing chondroitin sulphate and heparan sulphate have been studied physicochemically.

(1) Chondroitin sulphate protein was isolated from human aorta by Buddecke and Schubert (1961) and Buddecke et al. (1963) using the cetylpyridinium chloride method as described by Buddecke (1960). The purified product contained 19 percent protein, purified 22.6 percent hexosamine and 25.2 percent uronic acid and was thus similar in chemical composition to the preparation obtained by Buddecke et al. (1963) from bovine nasal cartilage. However, a physical examination showed that the aorta chondroitin sulphate protein molecule was significantly smaller than the bovine nasal cartilage proteinpolysaccharide molecule. The weight-average molecular weight reported by these workers was 9.5×10^4 as determined by light scattering and 15.0×10^4 as determined from sedimentation and diffusion data. Further, an examination of the infrared spectrum showed that the chondroitin sulphate was not chondroitin 4-sulphate such as is found in nasal cartilage but rather chondroitin 6-sulphate.

(2) Heparan sulphate protein has been

isolated from human aorta by Muir (1965) and Knecht et al. (1967) after enzymic digestion of the tissue. Chemical analysis performed on the preparations of these two groups of workers gave similar results, i.e. the product was a glycosaminoglycuronoglycan protein with a sulphate to hexosamine ratio of about 0.5 to 0.6 and with slightly less than one mole of acetyl per mole of hexosamine. Knecht et al. (1967) were able to demonstrate the presence of serine:xylose:galactose in the ratio of 1:1:2 in the carbohydrate-protein linkage region and have concluded that the linkage is similar to that found in bovine nasal cartilage proteinpolysaccharide.

The molecular weight of a heparan sulphate chain was estimated by Knecht et al. (1967) to be of the order of 2.5×10^4, which is similar in magnitude to the reported values for the chondroitin sulphate chains in bovine nasal cartilage preparations (table 1:2).

Because of the very limited studies that have been carried out on the glycosaminoglycuronoglycan protein complex of aorta it is not possible to draw any conclusions regarding its physicochemical characterization. However, the dearth of literature in this field has stressed the urgent need to further investigate

the physical and chemical composition of this complex in healthy and diseased tissues so that we may better understand the pathogenesis of arterial diseases such as arteriosclerosis.

I:3:4:2. The Glycoprotein Material from Aorta.

The glycoprotein material when isolated from aorta is considerably heterogeneous (Radhakrishnamurthy, Fishkin, Dessauer and Berenson, 1964; Radhakrishnamurthy et al., 1964; Kind et al., 1964; Berenson et al., 1966).

Radhakrishnamurthy et al. (1964) have isolated glycoprotein material from bovine aorta by using an ammonium sulphate fractionation. Subsequently, they purified a single glycoprotein species by electrophoresis on starch and polyacrylamide gels. Several components were observed in the electrophoresis on polyacrylamide gel, but two-thirds of the material was contained in a single band. Chemical analysis of this component which represented 0.3 percent of the original wet weight of the tissue showed the product to contain 79 percent protein, 5.2 percent carbohydrate (as determined by the phenol-sulphuric acid method), 3.5 percent hexosamine and 2.4 percent sialic acid. No uronic acid could be detected. This material then, is similar to the

glycoprotein materials isolated from other sources (Spiro, 1960; Berenson, 1961; Bourrillon and Got, 1962; Das, 1962; Eylar, 1965).

As yet few attempts have been aimed at a thorough examination of the physical properties of the members of this glycoprotein family. Radhakrishnam murthy et al. 1964) have made a limited study of the sedimentation characteristics of a one percent (w/v) solution of their preparation and have reported a sedimentation coefficient of 4.6 S. From this value, and the sedimentation coefficients of proteins of known molecular weight, these workers have estimated a molecular weight in the region 6 - 7 x 10^4. Kind et al. (1964) have reported a much higher molecular weight for their glycoprotein preparation which was purified on Sephadex G-200, i.e. 2.0 x 10^5. Barnes (1965) has extracted a 'sialoprotein' by alkali treatment of the aorta and after further treatment with proteolytic enzymes and chromatography on Sephadex G-50 has estimated the molecular weight of the carbohydrate residue to be of the order 6.3 - 6.4 x 10^3. Barnes (1965) has reasoned that since the total carbohydrate content of this preparation was approximately 8 percent the molecular weight of the 'sialoprotein' would be of

the order 8 x 10^4 which is similar to that estimated by Radhakrishnamurthy et al. (1964).

Radhakrishnamurthy and Berenson (1966) have presented evidence to show that a highly purified glycoprotein from bovine aorta contains two glycopeptides which have different carbohydrate and amino acid compositions. The molecular weights of the glycopeptides which these workers isolated were estimated as 2,300 and 1,500 respectively. They considered that if their previously estimated molecular weight of the glycoprotein was assumed (6 - 7 x 10^4, Radhakrishnamurthy et al., 1964) then it was possible that the glycoprotein could contain in its molecule four chains similar to the larger molecular weight glycopeptide and two chains similar to that of the smaller molecular weight glycopeptide.

I:3:5. CONCLUDING REMARKS AND DIRECTION OF THIS RESEARCH

The glycosaminoglycuronoglycans and the glycosaminoglycans are present in the ground substance of connective tissue in firm chemical combination with non-collagenous protein (Partridge and Davis, 1958). Hyaline cartilage has been frequently used for the characterization of the proteinpolysaccharide macromolecule, since it has a relatively simple carbohydrate

composition. In a few instances the proteinpolysaccharides present in other forms of connective tissue have been investigated, for example the hyaluronic acid protein of synovial fluid (Preston, Davies and Ogston, 1965), the chondroitin sulphate protein of synovial fluid (Silpananta et al., 1967) and the chondroitin sulphate protein of aorta (Buddecke et al., 1963).

The proteinpolysaccharide from nasal cartilage is composed of several polysaccharide chains attached to a polypeptide backbone (Mathews and Lozaityte, 1958; Buddecke and coworkers, 1963, 1967; Partridge and coworkers, 1961, 1965, and 1966b; Luscombe and Phelps, 1967a, b). The carbohydrate moiety has been found to contain keratan sulphate in addition to chondroitin sulphate (Meyer et al., 1953; Partridge and Elsden, 1961; Gregory and Rodén, 1961). However, it is not known with any certainty whether both these carbohydrate species are contained within a single molecule or whether they are present in separate molecules. More recent work (Hoffman, Mashburn, Meyer and Anderson Brey, 1967; Hoffman, Mashburn and Meyer, 1967) would tend to indicate that the former situation is the more probable.

Evidence has been presented which suggests that a protein distinct from the protein core material

is involved in the molecular structure (Fitton Jackson, 1965; Partridge and coworkers, 1965, 1966b). Meyer, (1966b) postulates that separate molecular units of the proteinpolysaccharide are cemented together with basic protein, further, such protein might be analogous to that separated by Partridge and coworkers (1965, 1966b). Anderson (1961) and Luscombe and Phelps (1967b) suggest that glycoprotein material is involved in the integral structure of the proteinpolysaccharide macromolecule.

From aorta extracts only two glycosaminoglycuronoglycan proteins have been investigated, i.e. chondroitin sulphate protein and heparan sulphate protein. Although the chemical composition of the chondroitin sulphate protein is similar to that of the chondroitin sulphate protein preparation from nasal cartilage the molecular weight is different. Such findings are not surprising when it is considered that the properties of the chondroitin sulphate proteins isolated from other forms of cartilage and from other connective tissues vary widely. Table I:3 summarizes the properties of the chondroitin sulphate protein preparations from various sources. Aorta has a significantly smaller molecular weight (9×10^4) than the other preparations cited. Such observations stress

Table 1:3. Characteristics of the Chondroitin Sulphate Preparations obtained from various Connective Tissues. (Figures given are the average of those quoted by the various authors.)

CONNECTIVE TISSUE	PP	PRINCIPAL	PROTEIN		REF.
	10^{-4} x MW	CS FORM	PERCENT	10^{-4} x MW	
Bovine Nasal Cartilage	47	4	20	(9.0)	1.
	24	4	7	2.0	2.
Pig Laryngeal Cartilage	23	4	2	0.5	3.
Human Rib Cartilage	18	6	25	(4.5)	1.
Human aorta	9	6	19	(1.7)	1.
Synovial fluid	25	6	16	(3.9)	4.

Abbreviations: PP, proteinpolysaccharide; CS, chondroitin sulphate; MW, molecular weight.

REF. References:
1. Buddecke et al. (1963).
2. Partridge, (1966b).
3. Muir and Jacobs, (1967).
4. Silpananta et al. (1967).

the need for examining the preparations obtained from these different tissues under closely similar experimental conditions in order to establish, (a) the correctness or otherwise of the molecular weights shown in table I:3, (b) whether or not these molecules are composed of smaller molecular units such as the molecular unit isolated by Partridge (1966b) from bovine nasal cartilage or that obtained by Silpananta et al. (1967) from synovial fluid, or indeed if the molecular weight found by Buddecke et al. (1963) for aorta chondroitin sulphate is the true molecular weight of a basic molecular unit and (c) if smaller molecular units do occur, how and why are they aggregated when examined in isolated systems and do such aggregates exist in the native tissue. The answer to such questions should assist greatly in understanding the role played by the proteinpolysaccharides in the native tissue.

Glycoproteins have been isolated from aorta extract and a limited number of studies have been made of their physical properties. No such material has been reported in bovine nasal cartilage, although the presence of glycoprotein has been suggested by many authors.

Eylar (1965) has shown that in general, the

carbohydrate moieties of glycoproteins appear to have structural rather than functional significance. It could well be that the glycoprotein components present in the ground substance are implicated in the association of several different proteinpolysaccharide molecular species (or several similar proteinpolysaccharide molecular species) to form the observed three dimensional ordered structures such as are found in connective tissues.

Thus, if we are to understand better the role of these substances in the native tissue it is necessary that we study these substances (the various glycosaminoglycuronoglycanproteins and the glycoproteins), not only as isolated components but also in the presence of each other. To this end it is necessary that the techniques of extraction and isolation employed be so directed and that all species have similar extraction and purification histories, i.e. no one species is isolated at the cost of any other species. The work described in the first section of this thesis has laid stress on this problem, which has been approached in the following manner:

(1) The various methods of extraction and isolation of proteinpolysaccharide and of polysaccharides from

connective tissue have been evaluated and the methods that appeared to have some advantage were experimentally investigated. The results of these investigations have been presented in Chapter 3. Aorta extracts have been used in the investigation since they afford a system of proteinpolysaccharide material where many different glycosaminoglycuronoglycan proteins are present. The results of these investigations revealed that the conventional methods were unsuitable for the separation of the proteinpolysaccharide materials present in aorta. (2) In view of this situation a new method, that of isopycnic density gradient centrifugation was examined. The results are presented in chapter 4. It will be shown that when well-characterized samples of a glycoprotein and a glycosaminoglycuronoglycan protein were examined, a good separation could be obtained; when applied to the more complex mixture present in aorta extract, the method effected only a partial separation. (3) Chemical characterization of the various fractions obtained after aorta extracts had been centrifuged in density gradients and the various methods that were used in further separating the fractions are presented in chapter 5.

Attention was then directed toward the

investigation of the proteinpolysaccharide material present in nasal cartilage in the hope that the information gained might be usefully employed in assessing the significance of the measured properties obtained with a preparation of chondroitin sulphate protein from aorta. The design of the investigation was as follows:

(1) The proteinpolysaccharide was isolated and examined in the ultracentrifuge and observed to be heterogeneous, two boundaries of different sedimentation rates were always present. The methods employed in separating these components and a description of their chemical composition are presented in chapter 6.

(2) A description of the physical properties of these components as visualized by sedimentation and viscosity is presented in chapter 7.

Finally, in chapter 8, the significance of these findings with regard to the methods of isolation and characterization of the proteinpolysaccharides and the physiological functions of these proteinpolysaccharides in the connective tissue is discussed.

Chapter 2.

General Experimental

2:1.MATERIALS

2:1:1.REAGENTS.

Analytical reagent grade materials were used in all experiments, except in the preparative density gradient experiments, where laboratory grade caesium chloride was used.

2:1:2.STANDARD BUFFER SOLUTION.

This was a phosphate buffer of pH 6.75 and I 0.1. It contained 10 mmoles of KH_2PO_4, 10 mmoles K_2HPO_4 and 60 mmoles of KCl per litre of solution. At 20° its density and viscosity relative to water were 1.0037 g per ml and 1.0049, respectively.

2:1:3.DIALYSIS TUBING.

This was cellulose casing dialysis tubing (Visking Co.Chicago, Illinois). Prior to use the tubing was successively soaked for 24 hours in 10 percent acetic acid, 24 hours in 10 percent sodium bicarbonate and rinsed repeatedly with distilled water and finally stored at 4° in 70 percent ethanol.

2:1:4.BIOLOGICAL MATERIALS.

The biological materials used were, bovine nasal cartilage and porcine aorta (the uppermost six inches of the descending thoracic section).

2:1:4:1. Extraction of the Proteinpolysaccharides from Aorta.

The intima was dissected from the remaining tissue and the proteinpolysaccharide material extracted into water by homogenizing in a VirTis 45 homogenizer (Research Equipment, Gardiner, New York). Several modifications were made to the methods of extraction and isolation and these will be discussed fully in chapter 3.

2:1:4:2. Extraction of the Proteinpolysaccharide from Cartilage.

Bovine nasal cartilage was cleaned of extraneous tissue, finely minced and immediately frozen (-20°). The time taken from the death of the animal to the freezing seldom exceeded two hours. The procedure used in the extraction and isolation of the proteinpoly - saccharide is shown in scheme 2:1.

2:1:4:3 Preparation of the Proteinpolysaccharide Solutions for Physical and Chemical Analysis.

Solutions for physical or chemical analysis were always diluted by weighing. They were allowed to stand for 16 hours before use, except when required for viscosity experiments, in which case they were used almost immediately.

SCHEME 2:1. Extraction and Isolation of the Protein - polysaccharide Material from Bovine Nasal Cartilage.

FROZEN MINCED CARTILAGE

Homogenized, 15 min, VirTis 45, (10 g per 200 ml H_2O)
Homogenate dialysed against several changes H_2O, 24 hours, 4°.
Centrifuge 21,000 rpm, 60 min.

SUPERNATANT	RESIDUE
Two volumes ethanol added. Centrifuge 9,000 rpm, 40 min.	Discarded-
SUPERNATANT	RESIDUE
Potassium acetate added to conc. 20 g per 1. Centrifuge 2,300 rpm, 20 min.	If any residue was present it was discarded
SUPERNATANT	RESIDUE
Discarded.	Suspended in H_2O. Dialysed against H_2O 24 hours, 4°. Two volumes ethanol added plus potassium acetate to 20 g per 1. Centrifuge 2,300 rpm.
SUPERNATANT	RESIDUE
Discarded.	Crude Proteinpolysaccharide Suspended in H_2O. Dialysed first against water and then against the standard buffer, 4°. Stored at -20°.

Centrifugation at 2,300 rpm, International Model PR2, rotor 276.
Centrifugation at 9,000 rpm, Servall Model RC2, rotor GSA.
Centrifugation at 20,000 rpm, Beckman-Spinco Model L, ultracentrifuge, rotor 21.

2:2. ANALYTICAL METHODS.

In this section and the succeeding one, only those methods which have been used throughout the work will be described. Other techniques and methods, for example density gradient centrifugation, will be described in the chapters where they have been most extensively used.

2:2:1.DETERMINATION OF CONCENTRATION OF SOLUTIONS.

The ultimate method was the determination of the dry weight of a solution that had previously been exhaustively dialysed against distilled water. The samples (3 to 4 ml containing 1 to 3 mg) were dried to constant weight at 105°. The weighings were made on a Mettler micro - analytical balance (model M5). Samples were weighed at room temperature. However, in order to obtain reproducible measurements it was found necessary to weigh the samples on consecutive days at approximately the same hour. Readings obtained were accurate to ± 5 micrograms.

2:2:2.URONIC ACID.

This was determined by the carbazole method of Dische (1947), using D-glucuronic acid (Sigma) as standard and Analytical reagent grade sulphuric acid (Ajax Chemical Co). Other samples of sulphuric acid (e.g. British Drug Houses Analar) were found to be unsatisfactory. After heating for 20 min, cooling and then adding the

carbazole reagent, the solutions were allowed to stand for 4 hours before being read in a Unicam SP 600 spectrophotometer at 530 mμ.

2:2:3.GLUCURONIC ACID: IDURONIC ACID RATIO.

These were determined using the method of Radhakrishnamurthy and Berenson (1963). The ratio was determined from the colour response after heating the hexuronic acid mixtures at several different temperatures. Previously characterized samples of chondroitin 4-sulphate and dermatan sulphate (Dunstone, 1962) were used as standards.

2:2:4.SIALIC ACID.

This was determined by the thiobarbituric acid method (Warren, 1959). The samples were first hydrolysed with 0.05 M H_2SO_4 for one hour at 80°. N-acetyl neuraminic acid (crystalline type II from egg or synthetic material, Sigma) was used as a standard.

2:2:5.HEXOSE.

This was determined by the phenol-sulphuric acid method (Dubois, Gilles, Hamilton, Rebers and Smith, 1956). D-galactose (British Drug Houses Ltd) was used as a standard. The solutions were read in a Unicam SP 600 spectrophotometer at 485 mμ. When necessary values were corrected for the presence of uronic acid, which gave a colour yield approximately one-third of that of an equal amount of galactose.

2:2:6.HEXOSAMINES.

Preparations were hydrolysed with 4N HCl for 8 hours at 100° in Quickfit stoppered tubes. Total hexosamine was determined by a modification (Cessi and Piliego, 1960) of the Elson and Morgan (1933) procedure. Galactosamine was determined according to the Cessi and Serafini-Cessi (1963) method. D-glucosamine HCl (Sigma) and D-galactosamine HCl (Sigma) were used as standards. Both of these compounds were at least 97 percent pure as measured by the ninhydrin method (Moore, Spackman and Stein, 1958) with L-alanine (Fluka, Puriss CHR grade)as standard.

2:2:7.PROTEIN.

This was determined by the method of Lowry, Rosebrough, Farr and Randall (1951) with bovine serum albumin (Sigma) as standard.

When only relative protein values were required, protein was estimated from the differences between extinctions at 215 mμ and 225 mμ (Murphy and Kies, 1960), or simply from the extinction at 210 mμ (Tombs, Souter and Maclagen, 1959). The extinctions were measured using a Zeiss (Model PMQ II) spectrophotometer.

2:2:8.SULPHATE.

This was estimated by the turbidimetric method of Dodgson and Price (1962), after liberation of the sulphate from the samples by hydrolysis in IN HCl for five hours. The modified Method B of Dodgson (1961) was used to accommodate smaller volumes of starting material. Potassium sulphate in IN HCl was used to obtain a calibration curve. Samples were compared in the Beckman DU spectrophotometer(German Model G 4700) using 10mm quartz cuvettes (Hellma No. 104-OS).

2:2:9.AMINO ACIDS.

Preparations were hydrolysed in 6N HCl for 22 hours. The HCl was removed under reduced pressure. Hydrolysates were redissolved and run on a Beckman-Spinco amino-acid analyser by Dr. D.C.Shaw. No correction was made for the destruction of certain amino acids that often occurs during acid hydrolysis of carbohydrate - containing materials (Pusztai and Morgan, 1964; Anderson etal., 1965).

2:2:10.INFRARED SPECTRA.

These were measured by Dr. E.Spinner with a Perkin-Elmer model 621 dual-grating instrument. One mg samples of lyophilized material were prepared in KBr disks.

2:2:11.COLUMN CHROMATOGRAPHY.

Jacketted chromatographic columns, designed for use with a refrigerated automatic fraction collector (Multi-column, Patons Industries Ltd., Beaumont, S.A.), were used.

2:2:11:1.DEAE-Cellulose and ECTEOLA-Cellulose.

DEAE-cellulose (Schleicher and Schuell, Keene, U.S.A.) and ECTEOLA-cellulose (Schleicher and Schuell, Keene, U.S.A.) were prepared for column chromatography according to the manner described by Pusztai and Morgan (1964) for DEAE-cellulose.

2:2:11:2.Cellulose Powder.

Cellulose powder (Whatman, standard grade) was prepared by washing the powder several times in distilled water and then equilibrating with the desired solvent.

Further details pertaining to the chromatographic procedures will be given in the text.

2:2:12.PARTITION CHROMATOGRAPHY ON PAPER.

The method was used only to identify sugars in a carbohydrate mixture. Samples were hydrolysed for 7 hours in 2N HCl at 100°, dried *in vacuo*, dissolved in water and then redried. The samples were then taken up

in a small quantity of water and applied to the chromatogram. The chromatogram was developed with butanol: pyridine:water (6:4:3) by the descending technique and stained with aniline hydrogen phthalate in the manner described by Wilson (1959).

2:2:13.POLYACRYLAMIDE GEL ELECTROPHORESIS.

The method as outlined by Ornstein and Davis (1962) was used. The runs, unless otherwise stated, were carried out in Tris-glycine buffer (pH 8.4) at 50 volts and 4 ma for approximately 150 min. Samples containing 25 to 50 µg of protein in 0.2 ml of 50 percent sucrose (w/v) were applied to 10 percent gel columns with 6 percent gel overlays; the columns were stained with Amido black.

2:3.PHYSICAL METHODS.

2:3:1.ANALYTICAL SEDIMENTATION.

Experiments were performed in Beckman-Spinco Model E ultracentrifuges (serial Nos. 358 and 494), equipped with rotor temperature and indicator control units. Schlieren optics were used on all occasions as was the An-E rotor. The speed, unless otherwise stated was 50,740 rpm. Usually two solutions were observed in each run; cells with 30 mm single sector aluminium centre - pieces were used. As most runs were of short duration the temperature was not controlled. The recorded

temperatures during a run seldom varied by more than 0.5°.

The rate of boundary movement through the cell was measured directly from the photographic plates (Ilford R40) using a two-coordinate microcomparator (Gaertner Toolmakers microscope, type M2001, AS-P). The sedi - mentation coefficients were then evaluated from the slope of the line relating the logarithm of the boundary position to time. i.e.

$$s = \frac{1}{\omega^2} \cdot \frac{d \ln x}{dt} \qquad \text{-------(2:1)}$$

where s is the sedimentation coefficient

ω is the angular velocity in radians per sec

x is the distance of the boundary in cm from the axis of rotation. As the boundaries were usually hypersharp, the position of the boundary was taken as that of the maximum ordinate.

t is the time in sec.

In computing the slope of the line the data was fitted using the method of least squares.

The observed sedimentation coefficient (s_{ob}) was corrected to 20° in water according to the relationship given by Svedberg and Pedersen (1940)

$$S_{20,w} = S_{obs} \frac{\eta t}{\eta_{20}} \frac{\eta}{\eta_0} \left(\frac{1 - \bar{v}\rho_{20,w}}{1 - \bar{v}\rho t}\right) \quad \ldots\ldots\ldots\ldots \quad (2:2)$$

where $s_{20,w}$ is the sedimentation coefficient at 20° in water

$\eta t/\eta_{20}$ is the viscosity of water at t° relative to that at 20°

η/η_0 is the relative viscosity of the solvent to that of water

$\rho_{20,w}$ is the density of water at 20°

ρt is the density of solvent at t°

$\bar{v}$ is the partial specific volume of the solute.

The partial specific volume $\bar{v}$ was taken as 0.55 (Mathews and Lozaityte, 1958). This value is in close agreement with the values quoted by other workers (Webber and Bayley, 1956; Buddecke et al., 1963) for the partial specific volume of proteinpolysaccharide preparations from bovine nasal cartilage.

When material sedimented as a single boundary correction to the concentration was made for sectorial dilution according to the equation given by Trautman and Schumaker (1954)

$$\frac{c_t}{c_o} = \left|\frac{x_o}{x_t}\right|^2 \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots \quad (2:3)$$

where c_0 is the initial concentration of the solution in g per dl

c_t is the concentration of the solution in the plateau region in g per dl at time t

x_0 is the position of the boundary at zero time (meniscus)

x_t is the position of the boundary at time t.

In making this correction, the concentration midway through the experiment was calculated. This calculated value then represents the average concentration during a run.

2:3:2.VISCOSITY.

These were determined using capillary Ostwald viscometers of the type described by Schachman (1957). 2 ml volumes were used on all occasions. The flow time (the time taken for the liquid to flow past two etched marks, above and below the bulb) was measured to within 0.1 sec with a stop watch. The flow time of the solvent, i.e. standard buffer, was approximately 100 sec at 25°. The temperature was controlled to $\pm$ 0.005° during any series of measurements by immersing the viscometer in a thermostated bath (model E 270, Townson and Mercer).

2;3;2;1.Preparation of Solutions.

Water, acetone and solvent were filtered twice through sintered glass filters (porosity 4). The water and solvent were then further cleaned by passing twice through Millipore filters (HA 0.45 μ, Millipore Filter Corporation, Bedford, Mass. U.S.A.). Acetone, used in drying the viscometers could not be further cleaned in this way, since it dissolved the Millipore filters. The cleaned solutions were stored in polythene bottles that had previously been exhaustively washed with the filtered solutions.

Dust and contaminating particles were removed from the proteinpolysaccharide solutions by centrifuging for 60 min at 40,000 rpm in the model L ultracentrifuge, rotor No.40. Dilutions of the supernatant were made with the filtered standard buffer.

2:3:2:2.Cleaning of Viscometers.

The viscometers were allowed to stand for two to four hours immersed in chromic acid before being washed exhaustively with filtered water and finally dried with filtered acetone. The viscometers were cleaned after every measurement.

2:3:2:3.Couette Viscometry.

A limited number of observations were made using a Couette viscometer, in order that the presence or absence of non-Newtonian viscosity might be detected. The determinations were carried out by Dr. P.Silpananta, using the apparatus described by Preston et al. (1965).

2:3:2:4.Calculations.

Results of the viscosity measurements were expressed in terms of the reduced viscosity,

$$\eta red = \frac{\eta sp}{c} = \frac{t/to - 1}{c} = \frac{\eta rel - 1}{c} \quad .....(2:4)$$

where c is the concentration of proteinpolysaccharide, g per dl

t is the flow time of the proteinpolysaccharide solution in sec

t_o is the flow time of the solvent in sec

ηrel is the relative viscosity i.e. t/t_o

ηsp is the specific viscosity i.e. $(t/t_o - 1)$

ηred is the reduced viscosity.

Numerous empirical equations have been proposed to express the viscosity as a function of concentration in order that the intrinsic viscosity might be determined.

The intrinsic viscosity [η] is defined by the relation

$$[\eta] = \lim_{c \to 0} \frac{\eta sp}{c} \qquad \text{.....(2:5)}$$

In this work two relationships have been used to determine the intrinsic viscosity

(a) The Huggins (1942) equation

$$\eta sp/c = [\eta] + k'[\eta]^2 c \qquad \text{.....(2:6)}$$

where ηsp/c is plotted against c and extrapolated to c=0 to give the intrinsic viscosity as the value of ηsp/c at c = 0

and

(b) The Kraemer (1938) equation

$$(\ln \eta rel)/c = [\eta] - k'' [\eta]^2 c \qquad \text{.....(2:7)}$$

where (lnηrel)/c is plotted against c and extrapolated to c = 0 to give the intrinsic viscosity as the value of (lnηrel)/c at c = 0.

The use of the two methods enables the intrinsic viscosity to be determined with more confidence.

By expanding (lnηrel)/c into a power series and combining the expanded equation and equation 2:6 we have (Yang, 1961)

$$k' + k'' = 0.5 \qquad \text{.....(2:8)}$$

and the comparison of measured values for k' and k" provide an additional check of the experimental procedure.

In cases where straight lines best fitted the experimental data least square lines were computed.

No correction was made to the reduced viscosity to allow for kinetic energy changes. Such changes were minimized by keeping the capillary diameter small (approximately 0.055 cm) and the capillary length large (approximately 50.0 cm) (Yang, 1961). In this way the error due to kinetic energy was always less than 0.1 percent.

Chapter 3.

An Evaluation of Some of the Methods Employed in the Extraction, Isolation and Purification of the Proteinpolysaccharides from Connective Tissue.

3:1. INTRODUCTION.

Separation of the proteinpolysaccharides from their cellular matrix is difficult and even with high speed homogenization it is impossible to extract all the carbohydrate-containing material. Further, prolonged periods of extraction may result in considerable degradation of the macromolecules due to the presence of proteolytic enzymes arising either from the natural cathepsins of the tissue or from contamination with micro-organisms (Partridge et al., 1965; Buddecke, 1966; Buddecke and Platt, 1966).

The isolation of the proteinpolysaccharides from connective tissue involves essentially three steps;

(a) homogenization of the tissue in an aqueous solvent to extract the proteinpolysaccharides into solution,

(b) removal of all collagen and other non-specific protein and isolation of the crude proteinpolysaccharide material and (c) separation and purification of the various proteinpolysaccharide species.

3:2. AN EVALUATION OF THE METHODS OF EXTRACTION ISOLATION AND PURIFICATION.

3:2:1.EXTRACTION.

Mechanical disintegration by means of high speed homogenization in aqueous solvents is presently the means used to extract the proteinpolysaccharides into solutions. Usually fresh tissue (Partridge et al., 1961) or acetone-dried preparations (Malawista and Schubert, 1958) are cut into small sections or finely minced, to facilitate homogenization. Not all the carbohydrate containing material is extracted in this way (Schubert, 1958; Lowther et al., 1967), however, increased yields may be obtained by repeated homogenization of the tissue and the use of increased volumes of extracting solvent (Schubert, 1958; Pal and Schubert, 1965).

Anderson (1961) has avoided long periods of homogenization and large volumes of extracting solvent by first disintegrating the material in a mill where the grinding chamber was maintained at -196°. By this means, cartilage could be disintegrated into particles, less than 1.6 mm in diameter, which readily dispersed in aqueous solution when warmed to 0°. That this method has not been more generally used, is due, possibly, to the lack of suitable equipment in many laboratories,

however, since the studies of Anderson (1961, 1962) were not concerned with retaining the macromolecular integrity, no attempt was made to determine whether or not this treatment resulted in degradation of the proteinpoly-saccharide material.

Cold water or cold dilute salt solutions are generally used as extracting solvents.

Berenson and Fishkin (1962) have made a limited number of observations with regard to the relative merits of water and 0.15M NaCI as extracting solvents for bovine aorta; they found that less uronic acid - containing material was extracted with water amd more glycoprotein material was extracted with salt.

A difference in the composition of the carbo-hydrate fractions isolated by different aqueous solvents has also been noted by Lowther et al. (1967). These workers found that the hyaluronic acid and 43 to 62 percent of the chondroitin sulphate were extracted with water whereas a further 38 to 57 percent of the chond-droitin sulphate material was extractable with salt solution.

Although water can cause significant disruption of the cells and cell particles and so release degradative

enzymes into the aqueous phase, it has been and still is the most widely used of all the extracting solvents, see for example the work of Buddecke and coworkers (1963, 1967), Luscombe and Phelps (1967a) and Lowther and Baxter (1966). Further, Hoffman et al. (1967) have extracted proteinpolysaccharide material from nasal cartilage under conditions in which the degradation of the proteinpolysaccharide by enzymes should be maximal; except for a small decrease in viscosity these workers found no difference between their product and similar material obtained from freshly killed animals and isolated as rapidly and efficiently as possible.

Snellman (1957) observed that tissue homogenates become acid very rapidly; he suggested therefore that buffered solvents should always be used. Such observations have not been reported by more recent workers. However, extremes of pH must be rigorously avoided since irreversible dissociation of the proteinpolysaccharide molecule is known to occur under such conditions (Partridge and Davis, 1958; Warner and Schubert, 1958; Hoffman et al., 1967). The proteinpolysaccharides in preparations subjected to such treatment cannot be considered in terms of their native state.

3:2:2. REMOVAL OF EXTRANEOUS PROTEIN AND ISOLATION OF THE PROTEINPOLYSACCHARIDES.

In general the method utilized in isolating the proteinpolysaccharide material from the homogenate will depend upon the form of the investigation. For struct - ural studies, such as the determination of the carbo - hydrate to protein linkage, treatment of the crude material with specific enzymes or with alkali, is permissible, since such studies do not require the macromolecular integrity to be maintained. When the investigations pertain to the macromolecular conform - ation, isolation is difficult, the nature of the ultimate product is obscure and only the well-defined precautionary methods can be applied.

Kaolin and Celite or similar preparations have been used on several occasions as absorbents for protein (Meyer and Chaffee, 1941; Einbinder and Schubert, 1951; Malawista and Schubert, 1958; Radhakrishnamurthy et al., (1964). Muir and coworkers, 1958, 1957). However, Snellman (1957) reported that proteins were not effectively removed in this way and if the extract is too heavily treated, losses of proteinpolysaccharide occur.

Several workers have removed collagen and other non-specific protein from their extracts by using reagents known to precipitate proteins, for example Muir and coworkers (1958, 1965, 1967) have reduced the protein content of their preparations by reacting the extract with 5-aminoacridine HCl which forms insoluble salts with sulphate esters (Dodgson, Rose and Spencer, 1954). Webber and Bayley (1956) on the other hand have removed the protein from the crude extract by the method of Meyer, Odier and Siegrist (1948) i.e. with HCl and phosphotungstic acid. Radhakrishnamurthy et al. (1964) have removed protein by precipitation at an acid pH and followed this with an ammonium sulphate fractionation. However, any method involving acid or alkaline conditions must be approached with caution especially if the proteinpolysaccharide is intended to be used for macromolecular studies.

The method most generally used is that of alcoholic precipitation of non-specific protein followed by precipitation of the proteinpolysaccharide material with ethanol and potassium acetate (Shatton and Schubert, 1954; Malawista and Schubert, 1958). To remove protein in this way, extracts must be thoroughly freed from salt before the addition of ethanol since, if salt is

present, proteinpolysaccharide material will also precipitate.

All collagenous material is not removed in this way and several extensions to the method have been employed. For example, the precipitation procedure may be repeated several times until all the collagenous material has been removed. Alternatively, Partridge et al. (1961) have stirred Celite with the preparation after the addition of ethanol and in some cases further purified the product by treating with ion-exchange resins. Gerber et al. (1960) found that material that had been isolated by precipitation with ethanol and potassium acetate could be purified simply by centrifuging at 78,000 x g for two hours. The supernatant was composed only of proteinpolysaccharide material while the residue contained both protein - polysaccharide and collagen (Scheinthal and Schubert, 1963).

The method of centrifuging to remove the extraneous protein has now been used by several other workers, for example Fitton Jackson (1965) and Serafini-Fracassini and Smith (1966). This method is mild and simple to perform and has also provided a uniform basis on which to compare the products obtained by several workers.

3:2:3.SEPARATION AND PURIFICATION.

The crude extract of proteinpolysaccharides obtained by the isolation methods described above will, in many cases, contain glycoprotein material in addition to glycosaminoglycuronoglycan protein. Thus, in considering a separation process for these materials it would seem feasible that these two groups of proteinpolysaccharides be separated before an attempt is made to separate the glycosaminoglycuronoglycan proteins. However, if consideration is to be given to the behaviour of these substances in the presence of each other, no one species should be isolated at the expense of any other.

3:2:3:1.Separation of Glycoprotein from Glycosaminoglycuronoglycan Protein.

Radhakrishnamurthy et al. (1964) have successfully isolated glycoprotein material from other glycosaminoglycuronoglycan protein material in bovine aorta by using an ammonium sulphate fractionation. However these authors have given no indication as to where in the fractionation process the glycosaminoglycuronoglycan proteins have been separated out. Nevertheless, this fractionation process could prove to be a useful method to employ in separating these materials.

Buddecke (1960) has reported a successful separation of the glycoprotein from the glycosaminoglycuronoglycan protein in papain digests of aorta by extraction with 90 to 95 percent phenol (w/v), a method originally described by Morgan and King (1943) for the isolation of blood-group glycoproteins. Buddecke (1960) and Buddecke and Schubert (1961) found that samples of chondroitin 4-sulphate and chondroitin 6-sulphate as well as bovine nasal cartilage proteinpolysaccharide were insoluble in aqueous phenol while glycoprotein material was not. However, Denborough and Ogston (1965) have attempted to use this method to remove the protein that remains associated with hyaluronic acid isolated from synovial fluid. The findings of these workers showed that phenol extraction reduced the protein content to, but not below, the value obtained by exhaustive filtration. The disparity in the findings of these two groups of workers might result from the relative complexity of aqueous extracts compared to enzymic digests such as used by Buddecke and coworkers (1960, 1961).

3;2;3;2.Separation and Purification of the Glycosamino-glycuronoglycan Proteins.

No method has been reported whereby several glycosamimoglycuronoglycan proteins present in a single system or preparation can be separated effectively. However, several methods have been described for the purification of preparations containing only one proteinpolysaccharide species or for the separation and isolation of several glycosaminoglycuronoglycans present in tissue extracts after proteolytic digestion. Two such methods will be discussed below.

(i). Chromatographic Methods.

Ringertz and Reichard (1960) have shown that it is possible to separate hyaluronic acid, chondroitin sulphate and heparin by chromatography on ECTEOLA-cellulose. A similar separation of hyaluronic acid and chondroitin sulphates from bovine skin and heart valves, previously treated with papain, has been reported by Toole, Goh, Lowther and Baxter (1965).

This latter group of workers have also used chromatography on ECTEOLA-cellulose to purify protein-polysaccharide preparations such as those from bovine nasal cartilage (Goh, Baxter and Lowther,1965), the hyaluronic acid protein from bovine skin (Lowther and Toole, 1965) and a chondroitin sulphate protein from

intervertebral disk (Lowther and Baxter, 1966).

Laurent (1961) has claimed that chromatography from columns of ECTEOLA-cellulose depends, in part, on a molecular weight fractionation; thus, fractions obtained from the column may contain materials that are chemically dissimilar and which overlap when eluted because of the polydisperse nature of the protein - polysaccharides in the original mixture (Northcote, 1966). Lowther and Toole (1965) found that when a glycosaminoglycuronoglycan protein mixture from bovine heart valves was chromatographed on ECTEOLA-cellulose two fractions were obtained and each fraction was a mixture of hyaluronic acid, chondroitin 4-sulphate, chondroitin 6-sulphate and dermatan sulphate. Such results could possibly be explained as a molecular weight rather than a chemical separation. However, Klynstra, van der Laan and Linders (1967) considered that chromatography on ECTEOLA-cellulose gave unreliable results for the separation of the glycosaminoglycurono-glycans and glycosaminoglycuronoglycan proteins from human aorta. These workers have attributed these findings to the interpenetration of the several maromolecular species present and suggest that the use of more dilute solutions might overcome this problem.

However, Antonopoulos, Fransson, Heinegard and Gardell (1967) are of the opinion that the particular 'batch' of ECTEOLA-cellulose greatly influences the separation that can be obtained. Thus, if careful consideration is given to the technical problems associated with column chromatography from ECTEOLA-cellulose columns a satisfactory separation of the glycosaminoglycurono-glycan proteins might be possible.

Chromatography from columns of DEAE-cellulose has been widely used for the separation and purification of glycoprotein material, see for example the work of Anantha, Natarajan and Cama (1965), Dunstone and Morgan (1965) and Kalzman and Eylar (1966). Barnes (1965) has reported separating glycoprotein material from glycos-aminoglycuronoglycan material present in alkaline extracts of aorta. This medium for chromatography might well be usefully employed in the separation and purification of glycosaminoglycuronoglycan proteins. Klynstra et al. (1967) have reported that DEAE-cellulose can also give unreliable results for the separation of glycosaminoglycuronoglycans and glycos-aminoglycuronoglycan proteins in aorta and it is possible that the same technical and experimental problems that are associated with ECTEOLA-cellulose are present when using DEAE-cellulose.

(ii) Separation and Purification using Quaternary Ammonium Ions.

Several workers have used this technique for the separation of polysaccharide mixtures isolated from connective tissues (Buddecke, 1960; Antonopoulos, Borelius, Gardell, Hamström and Scott, 1961; Antonopoulos et al., 1965; Antonopoulos, Gardell, Szirmai and de Tyssonsk, 1964; Szirmai, de Tyssonsk and Gardell, 1967; Thunell et al,, 1967; Dunstone, 1967). The method has also been comprehensively reviewed by Scott (1960).

The separation depends upon the formation of an insoluble complex between the organic polyanion(connective tissue polysaccharide) and the quaternary ammonium ion as follows:-

$$nRN^+ Cl^- + p^{n-} M^{n+} \longrightarrow (RN^+)_n p^{n-} + n(M^+ Cl^-)$$

where $RN^+ Cl^-$ represents the quaternary ammonium compound and $R^{n-} M^{n+}$ is the metal salt of the polyanion. Such complexes are insoluble in certain inorganic salt solutions provided the salt is present below a certain critical concentration.

Fractionation of the quaternary ammonium complex may be made in three ways (Northcote, 1966). (1) the polysaccharide and the quaternary ammonium compound are mixed in a salt solution sufficiently concentrated to prevent any of the polysaccharides from forming a complex.

Dilution of the salt solution enables the different polysaccharide-quaternary ammonium complexes to be precipitated sequentially in order depending on their solubility in the salt solution. In this way a minimum salt concentration required to dissolve a particular complex is obtained. This value is reproducible and characteristic of each polysaccharide (Scott, Gardell and Nilsson, 1957); (2) the polysaccharides may be precipitated in a solution of salt of a concentration sufficient to prevent precipitation of one or more of the components, while allowing others to precipitate completely (Scott, 1955); (3) all the polysaccharides are precipitated by the quaternary ammonium compound and the different complexes are then brought into solution by extracting the precipitate with solutions of increasing salt concentration(Antonpoulos et al., 1961). In this method it may be difficult to obtain a clear cut separation because of occlusion of soluble material within an insoluble "shell" of material with higher critical salt concentration. This difficulty is avoided if the complex is deposited as a thin layer on an inert support such as cellulose (Antonopoulos et al., 1961).

These methods have had a considerable degree of success in effecting separation of glycosaminoglycuronoglycans and glycosaminoglycans in artificial systems. Under these circumstances, glycoprotein and Keratan sulphate do not precipitate onto the cellulose (Buddecke, 1960) and thus can be removed simply by washing the cellulose with a solution of quaternary ammonium ions; hyaluronic acid, heparan sulphate, the chondroitin sulphates, dermatan sulphate and heparin are eluted from the cellulose by increasing the salt concentration. Although the method has been frequently applied to the separation of glycosaminoglycuronoglycans present in enzymic digests of connective tissue, the separation in these instances has been less successful; recent work has shown that it is difficult to obtain a clear cut separation of the glycosaminoglycuronoglycans from aortic tissue (Dunstone, 1967; Thunell et al., 1967).

The method has been used by several workers for the removal of unwanted protein and glycoprotein from their tissue homogenates (Buddecke and coworkers, 1963, 1967; Toole, Goh, Lowther and Baxter, 1965; Goh et al., 1965; Lowther and Baxter, 1966; Toole and Lowther, 1966; Luscombe and Phelps, 1967a). However, Buddecke et al. (1963) have shown that proteinpolysaccharide material purified in this way has a smaller molecular weight than

material that has not been subjected to such treatment. This could signify some change in macromolecular structure. On the other hand, Luscombe and Phelps (1967a) have found that such treatment does not appear to effect the chemical nature of the proteinpolysaccharide.

It would appear that this technique for separating glycosaminoglycuronoglycans would have only limited application in the separation of several glycosaminoglycuronoglycan proteins. It is possible however that it has application when the proteinpolysaccharide system is relatively simple i.e. when only one or two different proteinpolysaccharide types are present; it would not have application when a larger number of glycosaminoglycuronoglycan proteins are present together in a single system.

3:3. EXPERIMENTAL AND RESULTS.

The tissue used in these investigations was aorta, since, as has been repeatedly emphasized, this tissue provides a system that contains a wide spectrum of proteinpolysaccharides. In general the results of these investigations will be presented in a chronological order, however a division has been made between methods of extraction and isolation and the methods attempted in the separation and purification.

3;3;1.COLLECTION OF MATERIAL FROM THE ABATTOIRS.

Material was usually collected from the abattoir and transported back to the laboratory in crushed ice. It was felt however, that this might not completely eliminate the possibility of enzymic activity proceeding during this period. Thus material was placed into a vacuum flask containing solid CO_2 immediately upon the death of the animal and transported back to the lab - oratory. Specimens collected in this way were found to require too long a period to thaw-out sufficiently before the intima could be removed from them. In view of this the original method of transporting the specimens back to the laboratory in crushed ice was used.

3:3:2. INVESTIGATION OF EXTRACTION AND ISOLATION METHODS.

3:3:2:1. Method A.

In this method the intima, as soon as it was dissected from the remaining tissue, was frozen at -20° until sufficiently 'hard' to enable it to be minced (ordinary kitchen mincer). This procedure of freezing and mincing usually took several hours. The frozen minced intima was then homogenized in a VirTis '45' homogenizer for 30 min (10 g 'lots' each in 200 ml distilled water and the proteinpolysaccharide material isolated from the homogenate as outlined in scheme 3:1.

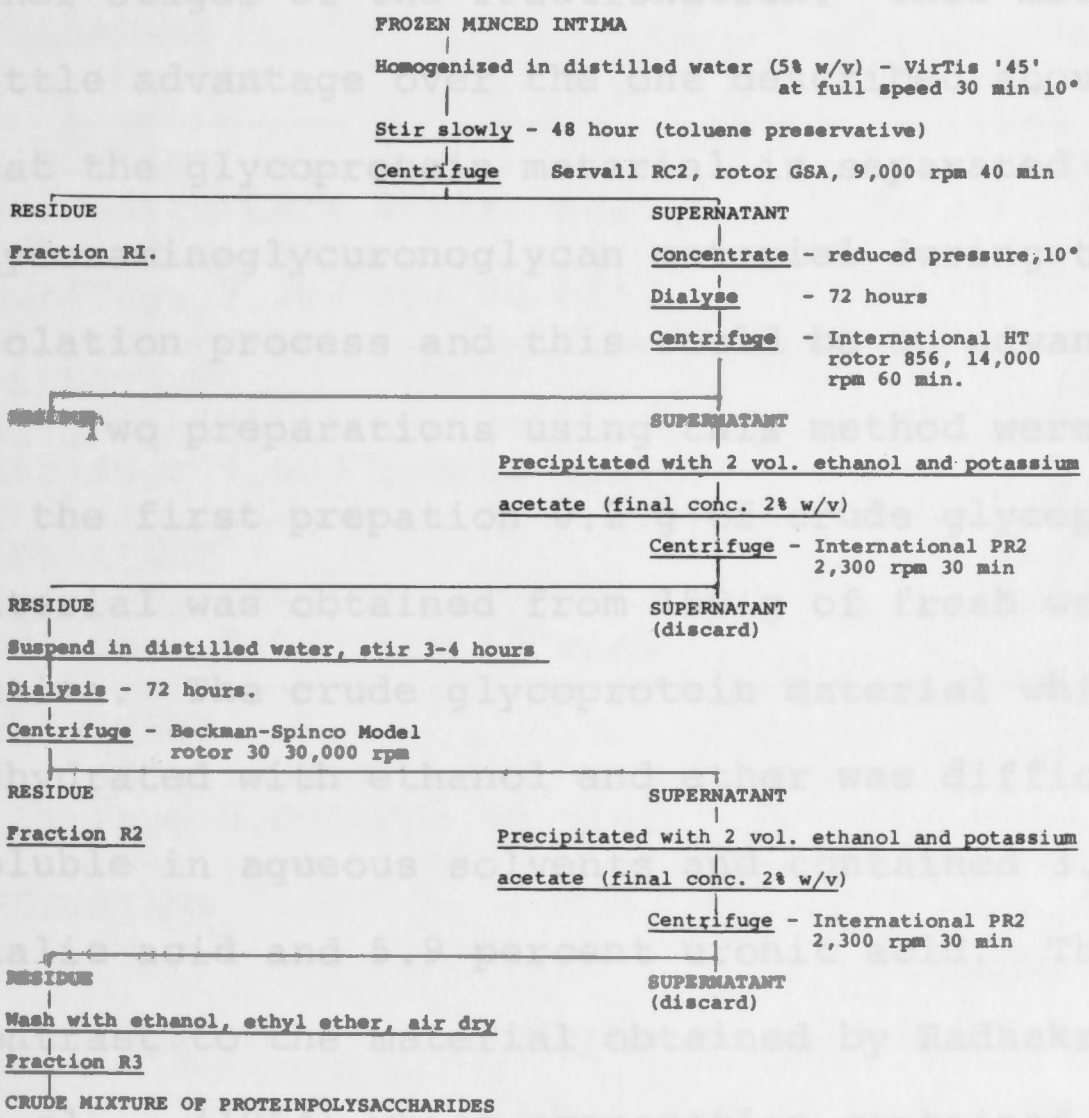
The distribution of tissue hexosamine between the various fractions resulting after the proteinpolysaccharide had been extracted in this way was: Fraction R1, 50 percent; Fraction R2, 10 percent and Fraction R3, 20 percent. A similar distribution of the uronic acid was obtained. Fraction R3 contained about 40 percent of the total sialic acid content and represented about 0.5 to 0.6 (w/w) of the original minced intima.

The main objection to this method of extraction and isolation was that it was considered to take far too long. Further, a considerable amount of the hexosamine material was not extracted from the intima (i.e. Fraction R1). More could be extracted if this residue was re-extracted with 1M NaCl, however this only increased the time of isolation. It was therefore decided to investigate other methods of extraction and isolation in the hope that the time required to isolate the proteinpolysaccharide material could be shortened.

3:3:2:2.Method B. (Method of Radhakrishnamurthy et al., 1964)

Radhakrishnamurthy et al. (1964) have extracted glycoprotein material from bovine aorta by homogenizing the aortas in 0.15 M NaCl, absorbing the non -specific protein on Celite, precipitating with acetate buffer and

Scheme 3:1. Isolation of the Proteinpolysaccharides from Porcine Aorta, Method A.



finally isolating the required material by an ammonium sulphate fractionation. An outline of the scheme is shown in scheme 3:2.

The crude glycoproteins were precipitated from solution with saturated ammonium sulphate while other carbohydrate containing materials were precipitated at other stages of the fractionation. This method has little advantage over the one described above except that the glycoprotein material is separated from the glycosaminoglycuronoglycan material during the isolation process and this could be an advantage.

Two preparations using this method were attempted. In the first prepation 0.2 g of crude glycoprotein material was obtained from 150 g of fresh wet minced intima. The crude glycoprotein material which was dehydrated with ethanol and ether was difficultly soluble in aqueous solvents and contained 3.4 percent sialic acid and 5.9 percent uronic acid. This is in contrast to the material obtained by Radhakrishnamurthy et al. (1964) whose preparation contained no uronic acid and was readily soluble in water and buffers. A second preparation was therefore attempted in which 205 g of wet minced intima was used. In this case no precipitate was observed when the solution was saturated

Scheme 3:2. Isolation of the Proteinpolysaccharides from Porcine Aorta, using the Method of Radhakrishnamurthy et al. (1964)

WET MINCED INTIMA. Extraction with 15 vols. 0.15 M NaCl. Centrifuged 2,300 rpm 20 min .

SUPERNATANT — RESIDUE

Dialysed against water
Centrifuged 9,000 rpm 60 min.

SUPERNATANT — RESIDUE

Filter Celite

SUPERNATANT — RESIDUE

Adjusted to pH 4.0 with acetate buffer, 0.01 M.
Centrifuge 2,300 rpm 20 min.

SUPERNATANT — RESIDUE

$(NH_4)_2SO_4$ 40% 16 hrs.
Centrifuge 9,000 rpm 60 min.

SUPERNATANT — RESIDUE

$(NH_4)_2SO_4$ 60% 16 hrs.
Centrifuge 9,000 rpm 60 min.

SUPERNATANT — RESIDUE

$(NH_4)_2SO_4$ 100% 48 hrs.
Centrifuge 9,000 rpm 60 min.

SUPERNATANT — RESIDUE

Crude Glycoprotein.

Centrifugation at 9,000 rpm; Servall RC2 centrifuge, GSA rotor.

Centrifugation at 2,300 rpm; International Model PR2 rotor 276.

with ammonium sulphate. Investigations were therefore carried out to see if the material had been precipitated out of solution at some earlier stage in the fraction - ation process. The material used in these investigations was a crude proteinpolysaccharide material prepared as in method A (section 3:3:2:1). This preparation contained both glycoprotein and glycosaminoglycuronoglycan protein as well as some non-specific protein. Two separate studies were made.

(i) Aliquots of fraction R3 were subjected to treatments with acid and buffer solutions to determine the advisability of using conditions of acid pH to remove non-specific protein.

Solutions of R3 were treated in the following manner:-

Solution 1, adjusted with concentrated HCl until concentration of HCl was 0.2M

Solution 2, adjusted with glacial acetic acid until the concentration of acetic acid was 0.1M

Solution 3, adjusted to pH 4.0 with 0.1M acetate buffer

Solution 4, adjusted to pH 4.0 with 0.01M acetate buffer

The solutions were allowed to stand for two hours before being centrifuged at 4,000 rpm (Martin Christ centrifuge model U.J.IS). Chemical analyses for uronic

acid, sialic acid and total carbohydrate (phenol sulphuric acid) were performed on each of the supernatants. The results of these analyses are shown in table 3:1.

Table 3:1. The effect of acid pH on the solubility of fraction R3. Details of the preparation and of the various acidic treatments are given in the text. Results are expressed as the percentage of material remaining in solution after treatment.

ANALYSIS	% OF MATERIAL REMAINING IN SOLUTION			
	I[a]	II[b]	III[c]	IV[d]
Uronic acid	63	68	6	3
Sialic acid	80	94	8	14
Total Carbohydrate	97	93	6	6

[a], 0.2M HCl; [b], 0.1M acetic acid; [c], 0.1M acetate buffer; [d], 0.01M acetate buffer.

The results in table 3:1 show that significant amounts of uronic acid-containing material are precipitated out from a solution containing aortic proteinpolysaccharides if that solution is adjusted to acid pH, further, up to 95 percent of uronic acid and sialic acid-containing materials are precipitated when the preparation is adjusted to pH 4.0 with either 0.1M

or 0.01M acetate buffer. This would indicate that both glycoprotein and glycosaminoglycuronoglycan protein materials can be co-precipitated with non-specific protein when the method of Radhakrishnamurthy et al. (1964) is used for the isolation of proteinpolysaccharides from aorta.

(ii) This investigation was aimed at determining if the ammonium sulphate fractionation was effective in the separation of sialic acid containing material from uronic acid containing material i.e. in the separation of glycoprotein from glycosaminoglycuronoglycan protein.

The details of the ammonium sulphate fractionation are shown in scheme 3:3. Chemical analysis was performed on the fractions P1, P2, and S2; the results of these analyses are given in table 3:2. There has been little separation of uronic acid and sialic acid-containing material during this fractionation.

Consideration of the findings from these two investigations suggest that the method of isolating glycoprotein material described by Radhakrishnamurthy et al. (1964) was unsuitable for isolating protein - polysaccharide materials from porcine aorta. Significant amounts of the glycosaminoglycuronoglycan protein and glycoprotein were coprecipitated with non-specific protein and the ammonium sulphate fractionation did not

Scheme 3:3. Ammonium Sulphate Fractionation of Fraction R3.

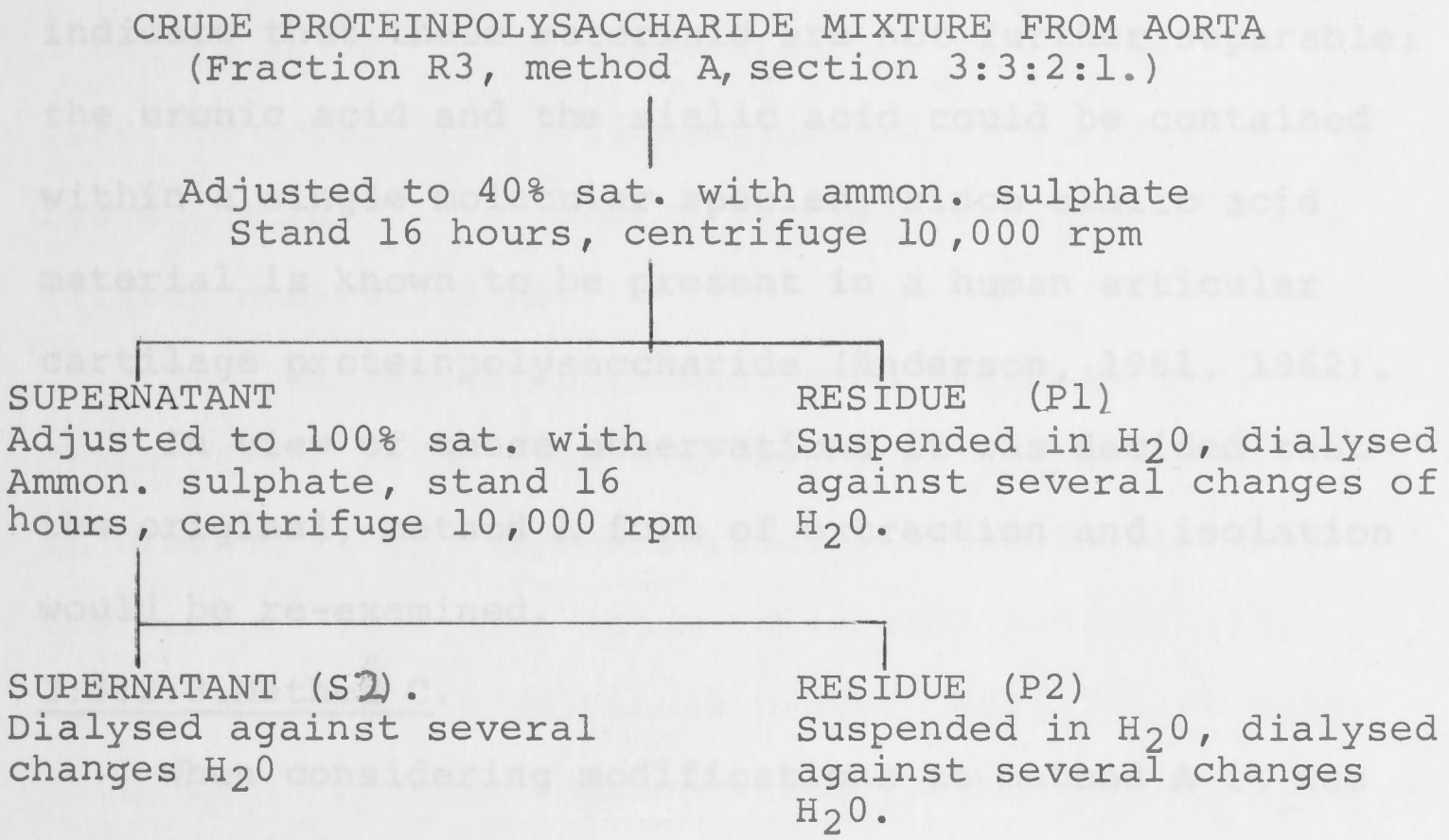


Table 3:2. Ammonium Sulphate Fractionation of Fraction R3. Details of the fractionation are given in scheme 3:4. The results are expressed as µg of uronic acid and sialic acid in each of the fractions.

FRACTIONS	URONIC ACID µg	SIALIC ACID µg	URONIC: SIALIC
P1	18.2	25.7	0.71
P2	71.5	65.3	1.09
S2	10.5	9.3	1.13

effectively separate the remaining uronic acid and sialic acid-containing materials. On the other hand, this could indicate that these materials are not further separable; the uronic acid and the sialic acid could be contained within a single molecular species, since sialic acid material is known to be present in a human articular cartilage proteinpolysaccharide (Anderson, 1961, 1962).

In view of these observations it was decided that the original, method A form of extraction and isolation would be re-examined.

3:3:2:3.Method C.

When considering modifications to method A it was kept in mind: (1) the proteinpolysaccharide material should be isolated in the fastest possible time following the death of the animal so as to avoid degradation from proteolytic enzymes (2) except with prolonged homegenization periods and large volumes of extracting solvent, it was extremely difficult to extract all the hexosamine containing material from the tissue and (3) dehydration of the preparation with ethanol and ether could result in some degradation. It was therefore decided that no attempt would be made at this stage to try and extract the maximum amount of proteinpolysaccharide from the tissue but rather, only

that material that could be extracted in the minimum of time would be used and further the preparation so obtained would not be dehydrated but stored in the solution form. The following method of extraction and isolation was the procedure adopted after considering several modifications.

Several aortas collected immediately upon the death of the animals were transported to the laboratory in crushed ice. They were removed sequentially from the vacuum flask, the intima dissected and placed immediately into a polythene beaker packed about with solid $C0_2$. As this tissue became frozen, (3 - 5 min), it was cut into small strips and placed in another beaker similary packed about with solid $C0_2$.

As soon as 10 g of sliced frozen intima became available it was homogenized in 200 ml of ice-cold distilled water in the VirTis '45' homogenizer for 10 min. Continuing in this way it was possible to have homogenized several 10 g 'lots' before the completion of the dissection process.

When sufficient homogenate became available it was centrifuged at 9,000 rpm for 40 min, (Servall RC2,GSA rotor), and to the supernatant was added two volumes of ice cold ethanol, followed by potassium acetate to a

concentration of 20 g per litre. The precipitate obtained was suspended in distilled water, stirred gently for 30 min (magnetic stirrer) and dialysed against several changes of distilled water for 20 hours, 4°.

Following this procedure it was possible to have completed the first alcoholic potassium acetate precipitation for all the specimens (usually about 70) within nine hours of the death of the first animal.

After dialysing for 20 hours the non-diffusible material was centrifuged for 40 min at 20,000 rpm (model L ultracentrifuge, rotor No. 21) and the crude proteinpolysaccharide material isolated by the addition of two volumes of ethanol and potassium acetate to 20 g per litre. The precipitate was taken up in water, stirred gently for 30 min, dialysed first against water and then against standard buffer and finally stored in solution at -20°. The whole procedure took, on the average, about 60 hours from the death of the first animal. An outline of the preparation is given in scheme 3:4.

The material isolated represented approximately 0.3 percent of the original wet intima weight. This is about half the material that was recovered using the

Scheme 3;4. Modified Method of Extracting Proteinpolysaccharides from Porcine Arterial tissue (method c).

FROZEN SLICED INTIMA
Homogenized in water
Centrifuge 9,000 rpm 10 min.

SUPERNATANT
two vols. ethanol
2 % pot. acetate
Centrifuge 2,300 rpm

RESIDUE

SUPERNATANT

RESIDUE
Suspended in water
Stirred 30 min.
Dialysed against water
16 hours.
Centrifuge 20,000 rpm

SUPERNATANT
two vols. ethanol
2 % pot. acetate.
Centrifuge 2,300 rpm

RESIDUE

SUPERNATANT

RESIDUE
Crude Proteinpolysaccharide

Suspended in water, stirred
30 min. Dialysed against
water and buffer. Stored -20°

previous method, (Method A, section 3;3;2:1) but in this case the material was obtained in at least one third of the time.

3:3:3. SEPARATION AND PURIFICATION.

The material used in these investigations was the crude proteinpolysaccharide material obtained using method A i.e. fraction R3, section 3:3:2:1. Initial experiments were designed to separate the glycoprotein material from the glycosaminoglycuronoglycan protein material, while later attempts were made to separate the various glycosaminoglycuronoglycan proteins.

3:3:3:1. Separation of Glycoprotein from Glycosaminoglycuronoglycan Proteins.

The method of Radhakrishnamurthy et al. (1964) has been shown to be unsuitable for the separation of the glycoproteins from the glycosaminoglycuronoglycan protein in porcine aorta. The method of phenol extraction was therefore investigated.

The procedure was carried out as described by Morgan and King (1943). In order to compare the behaviour of aortic proteinpolysaccharides with that of well characterized compounds, four different preparations were similarly treated i.e. a chondroitin 4-sulphate (Dunstone, 1962), a crude glycoprotein preparation (method B, section 3:3:2:2), a protein -

polysaccharide preparation from bovine nasal cartilage and the crude proteinpolysaccharide preparation from aorta (method A, section 3:3:2:1). Determination of the uronic acid, sialic acid and hexosamine were used to define the distribution of the material after the extraction. The results are shown in table 3:3.

Chondroitin 4-sulphate and the proteinpolysaccharide from bovine nasal cartilage are insoluble in aqueous phenol while the glycoprotein preparation is mainly phenol-soluble. Such findings are in agreement with the findings of Buddecke and coworkers (1960, 1961) and would suggest that the separation of similar components in aorta is probable. However, the results for the aorta proteinpolysaccharide preparation show an almost equal distribution of uronic acid-containing material and sialic acid-containing material between the phenol soluble and phenol insoluble fractions.

As the glycoprotein material contained in aorta is usually characterized by the presence of significant amounts of sialic acid and the absence of uronic acid (Radhakrishnamurthy et al., 1964) and the glycosaminoglycuronoglycan protein components are characterized by relatively large amounts of uronic acid and little or no sialic acid (Buddecke, 1960; Buddecke and

Table 3:3. Solubility in Aqueous Phenol of Aortic Proteinpolysaccharides. Samples of chondroitin 4-sulphate, chondroitin sulphate protein and glycoprotein have been included for comparison.

Chemical analysis of the samples. (g per 100 g of material)

SAMPLE	SIALIC ACID	URONIC ACID	HEXOSAMINE
Chondroitin 4-sulphate	0.1	33.0	28.0
Nasal Cartilage Proteinpoly-saccharide	0.8	28.0	28.0
Aorta Extract (fraction R3, Method A, 3:2:2:1)	2.0	6.0	7.0
Crude glycoprotein (method B, 3:2:2:2)	4.0	4.0	9.0

Distribution of sialic acid, uronic acid and hexosamine after the Phenol Extraction

(Results are expressed as the percentage of material extracted into each fraction).

(1) Phenol Soluble

SAMPLE	SIALIC ACID	URONIC ACID	HEXOSAMINE
Chondroitin 4-sulphate	-	<1	<1
Nasal Cartilage Proteinpoly-saccharide	-	<1	<1
Aorta Extract	38	51	54
Crude Glycoprotein	94	87	92

(2) Phenol Insoluble.

SAMPLE	SIALIC ACID	URONIC ACID	HEXOSAMINE
Chondroitin 4-sulphate	-	>99	>99
Nasal Cartilage Proteinpoly-saccharide	-	>99	>99
Aorta Extract	62	49	46
Crude Glycoprotein	6	13	8

Schubert, 1961) it was concluded that no significant separation of the glycoprotein from the glycosaminoglycuronoglycan protein had been obtained by the use of phenol and that both these types of proteinpolysaccharides contained phenol-soluble and phenol-insoluble components. These findings are at variance with those of Buddecke (1960) who found no phenol-insoluble glycoprotein and no phenol-soluble glycosaminoglycuronoglycan.

3:3:3:2. Separation of the Glycosaminoglycuronoglycan Proteins.

Two methods were investigated, namely that of chromatography on DEAE-cellulose and on ECTEOLA-cellulose and that of using quaternary ammonium ions.

(i) In the chromatographic experiments a column of 1.0 x 15.0 cm was used. ECTEOLA-cellulose and the DEAE-cellulose were equilibrated with 0.01M NaCl and material was eluted from the column by means of a continuous gradient of NaCl. In each case 5 mls of a 0.01 percent (w/v) solution of fraction R3, (section 3:3:2:1) was applied to the column. The flow rate was 25.4 ml per sq. cm per hour.

In the experiment using ECTEOLA-cellulose the proteinpolysaccharide material 'plugged up' the column

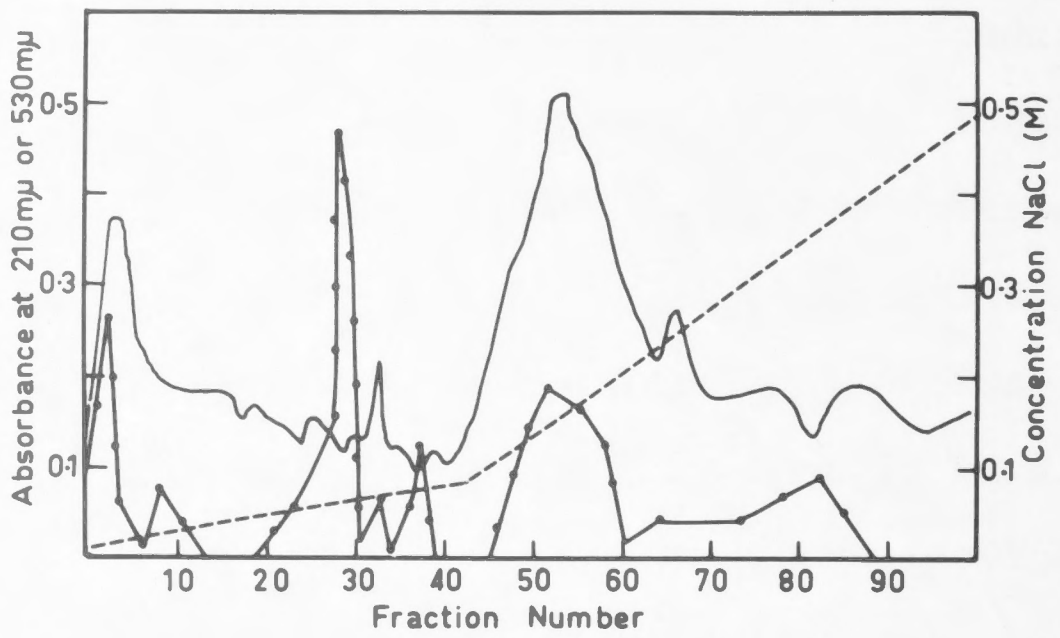


Fig. 3:1. Chromatography on DEAE-cellulose of a crude extract of proteinpolysaccharides from porcine aorta. Details of the preparation are given in the text. 5 ml of a 0.01 percent solution (w/v) was applied to the column (15.0 x 1.0 cm). Continuous elution is indicated by the line - - - - . The elution pattern was determined (a) by measuring the extinction of each fraction (10 ml) at 210 mμ, shown by the continuous line and (b) by determining the total carbohydrate content of each fraction (phenol sulphuric acid method) shown by the line ●—●—● .

and further elution was impossible. Further, at this stage, it was observed by Dr. J.R.Dunstone that a preparation of nasal cartilage proteinpolysaccharide that had been chromatographed on ECTEOLA-cellulose had a significantly smaller sedimentation coefficient than material that had not been so treated.

The behaviour of aortic proteinpolysaccharides on DEAE-cellulose was therefore investigated. The elution pattern obtained by reading the extinction of each fraction at 210 mμ and also by determining the total carbohydrate content of each fraction (phenol sulphuric acid method) is illustrated in fig 3:1).

Material eluted from the column can be grouped into three main fractions (Fraction Nos. 1-3, 20-30 and 41-64). However, there was no clear cut separation between the fractions.

The contents of the tubes containing the three main fractions were pooled, dialysed free of NaCl, concentrated by ultrafiltration and the uronic acid and sialic acid content of each determined. The results are shown in table 3:4.

Table 3:4. Chemical analysis of the pooled fractions after chromatography on DEAE-cellulose of Fraction R3. Details of the preparation are given in the text.

FRACTIONS POOLED	FRACTION NO.	URONIC ACID µg	SIALIC ACID µg
1 - 3	I	56.0	3.3
20 - 30	II	22.8	11.4
41 - 64	III	252.0	91.3
Initially		1190.0	280.0
Recovered		331.0	106.0
Percentage recovery		27.5	37.5

About one third of the material only was recovered from the DEAE-cellulose column and although a significant amount of material was contained in the fractions not pooled, it is doubtful if this could account for the remaining two-thirds of the material; thus it would appear that a large part of the proteinpolysaccharide material was not eluted from the column. In view of this and the fact that no definite separation of material had been achieved the method was considered unsuitable for the separation of the proteinpolysaccharides from aorta.

(ii) Separation Using Quaternary Ammonium Ions.

It has been shown that precipitation of polysaccharides onto cellulose using quaternary ammonium ions followed by elution of the cellulose with increasing concentration of salt results in at least a partial separation of the connective tissue polysaccharides. The method has also been used in the purification of single proteinpolysaccharide species but has not been used in the separation and purification of several proteinpolysaccharides present in a single system. Thus a solution of Fraction R3 obtained from porcine intima was used to test such a possibility.

A solution of Fraction R3 (method A, 3:3:2:1) was precipitated onto cellulose with cetylpyridinium chloride using the batch process described by Buddecke (1960). After standing for several hours material that had not precipitated onto the cellulose was removed and the remaining material eluted from the cellulose with increasing concentrations of $MgCl_2$. The proteinpolysaccharides were recovered from the several fractions as described by Antonopoulos et al. (1964). The uronic acid contained in each fraction was then estimated. The results are shown in table 3:5.

Table 3:5. Precipitation onto Cellulose with Cetylpyridinium Chloride of Fraction R3. Details of the preparation are given in the text.

ELUTING SOLVENT	URONIC ACID RECOVERED.(µg).
1 % Cetylpyridinium Chloride	1500
0.1 M $MgCl_2$	630
0.5 M $MgCl_2$	720
0.75 M $MgCl_2$	360
1.0 M $MgCl_2$	225
2.0 M $MgCl_2$	-
Uronic acid Initially	4540
Uronic acid Recovery	3435

Approximately 75 percent of the uronic acid containing material was recovered. However only 56 percent of this material was contained in the $MgCl_2$ fractions, 44 percent did not precipitate onto the cellulose. In view of these findings and also in view of the recent observations of Dunstone (1967) and Thunell et al. (1967) who found that it was extremely difficult to obtain a clear separation of the glycosaminoglycuronoglycans from aorta using this method, it was decided that this technique would not be further investigated until the proteinpolysaccharide components had been at least

partially fractionated.

3:3:4. CONCLUDING REMARKS.

In a search for a suitable method of separating the glycosaminoglycuronoglycan proteins of aorta the methods described in this chapter were investigated because they had been used successfully in the separating of glycosaminoglycuronoglycan mixtures. None of the methods attempted gave satisfactory separations of the protein-polysaccharide components of porcine aorta. The reasons for this may result from the relative complexity of aqueous extracts compared to enzymic digests. In aqueous extracts the components are probably present in a form more akin to their native state where interaction between the various molecular species would almost certainly occur. This would undoubtedly result in a modification of the properties of such preparations.

In the light of these findings it was necessary to search for some other technique to separate these proteinpolysaccharides. Consideration was therefore given to the various physical properties of these substances and this led to the suggestion by Dr. J.R. Dunstone that isopycnic density gradient centrifugation might be a useful technique to investigate in view of the differing partial specific volumes of these component materials.

Chapter 4.

Equilibrium Sedimentation of Protein - polysaccharides in a Density Gradient.

4:1. INTRODUCTION.

In recent years isopycnic density gradient centrifugation has become a valuable tool for the separation of macromolecules, particularly nucleic acids and cell particulates. The separation is based solely on the difference in buoyant densities of the various macrospecies. However as yet this technique has not been applied to the separation of the proteinpolysaccharide material such as found in connective tissue.

An aqueous extract obtained from connective tissue contains mainly proteinpolysaccharides and non-specific protein. The proteinpolysaccharide fraction is made up of glycosaminoglycuronoglycan protein and glycoprotein.

Glycosaminoglycuronoglycan protein material has a partial specific volume in the region of 0.57 ml per g (Mathews and Lozaityte, 1958; Buddecke et al., 1963; Tanford, Marler, Jury and Davidson, 1964) and thus should have a buoyant density near 1.75 g per ml. On the other hand glycoprotein material of the type described by Radhakrishnamurthy et al. (1964) by analogy with the glycoproteins isolated from other sources would be expected to have a partial specific volume of the order of 0.68 to 0.72 ml per g (Spiro, 1960; Bezkorovainy, 1965; Jamieson,1965; Williams and Peacocke, 1965) corresponding to a density near 1.45 g per ml.

Non-specific protein would be expected to have a density near 1.4 g per ml or less (Springall, 1954).

Extracts of aorta should provide a suitable proteinpolysaccharide complex on which to evaluate the application of isopycnic density gradient centrifugation to the separation of the proteinpolysaccharides of connective tissue.

4:1:1. DENSITY GRADIENT CENTRIFUGATION.

In density gradient centrifugation, substances are forced to migrate, by application of a centrifugal force, through a solution, in which the distribution of the solute has produced a density gradient; the density of the gradient increasing with increasing distance from the centre of rotation.

Density gradient experiments can be classified into three categories according to the way in which the gradient is used, namely, stabilized moving boundary centrifugation, zonal centrifugation and isopycnic gradient centrifugation.

Stabilized moving boundary centrifugation is analogous to classical ultracentrifugation, aimed at determining the sedimentation coefficients. Initially the macromolecules are uniformly distributed throughout the gradient forming material. The gradient is used

simply as a means of stabilizing (against convection and other anomalies) the boundaries formed during centrifugation. This permits centrifugation to be interrupted before complete sedimentation. The sample can then be separated into several fractions which are then analysed by suitable means to determine the position of the boundary.

Zonal centrifugation and isopycnic gradient centrifugation are high resolution techniques for the separation of macrospecies.

In zonal centrifugation, particles are separated into discrete zones on the basis of differences in their sedimentation rates i.e. upon the differences in molecular weight of the macrospecies and the difference in density between the particles and their supporting medium. With this method a solution containing particles of varied characteristics is layered onto the gradient column before centrifugation. Each substance sediments at its own rate and will form a band or zone in the fluid column. The zones of macrospecies will be separated from one another by distances related to their sedimentation rates. The gradient stabilizes the zones during centrifugation. Fractionation is then made by suitable means.

Separation by isopycnic gradient centrifugation is based solely on the difference in the buoyant densities of the macrospecies. In this method the initial position of the particles is unimportant. They may be uniformly distributed through the medium or they may be introduced as a discrete zone. The gradient may be preformed or allowed to develop during centrifugation. Sedimentation is continued until the particles reach an equilibrium position in the gradient, where the solution density is equal to that of the macrospecies.

4:1:2. EQUILIBRIUM SEDIMENTATION OF MACROMOLECULES IN A DENSITY GRADIENT.

The use of density gradient centrifugation for the separation of cell particulates with differing specific gravities has been described by several workers, for example Kuff and Schneider (1954), Blaschko, Hagen and Hagen (1957) and Barnett, Hagen and Lee (1958). The gradients in these instances were preformed and the material under investigation layered on top of the gradient column.

More recently, the method of isopycnic density gradient centrifugation, where the gradient is not preformed but allowed to develop during centrifugation, has been introduced (Meselson, Stahl and Vinograd, 1957).

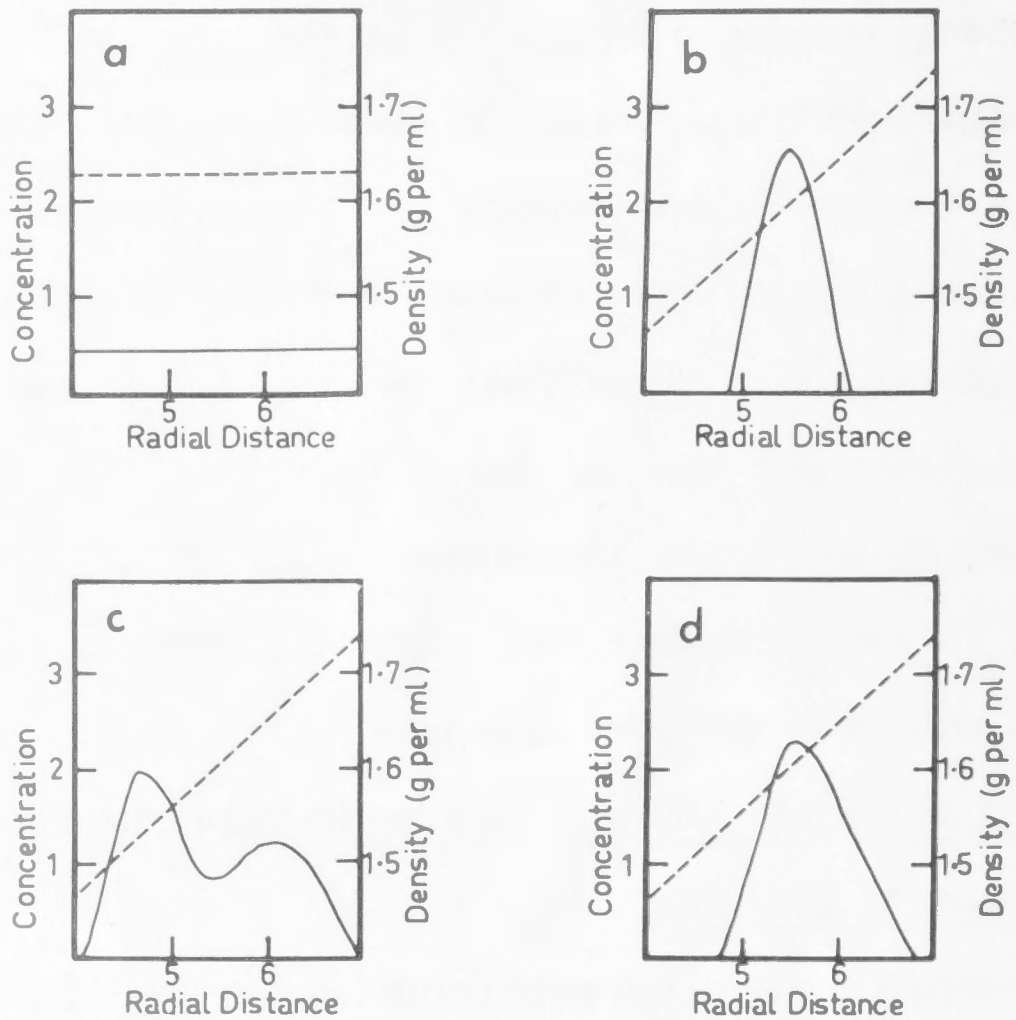


Fig. 4:1. Distribution with respect to concentration of macromolecules which have been centrifuged to equilibrium in a CsCl solution of initial density 1.63 g per ml. - - - , concentration of CsCl with respect to radial distance; ——— , concentration of macromolecules with respect to radial distance.
Situation before centrifugation, a) the macromolecules are uniformily distributed throughout the CsCl solution which has a uniform density of 1.63 g per ml.
Situation at equilibrium b) Gaussian distribution resulting when the solute is homogeneous and its behaviour ideal, c) bimodal distribution resulting when the solution of macro - molecules contains species with sufficiently different buoyant densities and d) skewed unimodal band indicating compositional heterogeneity.

In this method, the macromolecules are dissolved in a concentrated solution of a low molecular weight solute, the initial density of which is selected to correspond approximately with the buoyant density of the macrospecies. As centrifugation proceeds the low molecular weight solute will sediment to form a density gradient, the density decreasing at the meniscus and increasing at the cell bottom. This will cause the macromolecules to sediment from the top and to float up from the bottom. This redistribution of macromolecules, continues from both ends until equilibrium is achieved, where no further changes in the distribution of the components occurs and the macromolecules have found positions of equal density. The solution density at this point is the same as the buoyant density of the macrospecies, which are distributed with respect to concentration in a band which has been shown to correspond to a Gaussian distribution, provided the solute is homogeneous and its behaviour ideal (Meselson et al., 1957; Vinograd and Hearst, 1962).

If the solution of macromolecues contains species of sufficiently different buoyant densities a bimodal or polymodal distribution may be observed. Skewed unimodal bands indicate heterogeneity in buoyant density, the skewness resulting from compositional heterogeneity.

If the buoyant density of the particles is greater than the maximum density of the gradient, they will all sediment to the cell bottom. Alternatively, if their buoyant densities are smaller than the minimum density of the gradient, they will float to the cell top.

A diagrammatic representation of the distributions referred to above are given in fig. 4:1.

Establishment of the gradient during centrifugation requires considerably more time for the system to reach equilibrium than does a preformed gradient. However the gradient so obtained is calculable in terms of thermodynamic equations and the properties of the molecules forming the gradient (Ifft, Voet and Vinograd, 1961); it is impossible to obtain an exact description of a preformed gradient at the end of centrifugation, unless true equilibrium has been reached.

De Duve, Berthet and Beaufay (1959) considered that the increased time involved by forming the gradient during centrifugation would make the method unsuitable to have application with fragile subcellular particles. However the method has been widely used in the characterisation of the nucleic acids (Meselson and Stahl, 1958; Rolfe and Meselson, 1958; Hearst and Vinograd, 1961; Hearst, Ifft and Vinograd, 1961; Vinograd and Hearst, 1962).

Further, the method is of considerable advantage when dealing with macrospecies which have only a limited solubility in aqueous solution, since it does not require the molecules to be layered onto the gradient in a concentrated zone.

4:1:3. EXPERIMENTAL PROCEDURES USED IN EQUILIBRIUM GRADIENT CENTRIFUGATION.

Experiments involving sedimentation equilibrium in a density gradient can be performed in both the analytical and the preparative ultracentrifuges; the resultant concentration distribution at equilibrium is independent of the shape of the container (Vinograd and Hearst, 1962). However to compensate for the increased density of the gradients the manufacturers usually recommend that all the rotors be run at lower speeds.

The experiments in the analytical ultracentrifuge are simple to perform but have limited application because only an optical record of the distribution is obtained. On the other hand using the preparative ultracentrifuge it is possible to isolate separate layers of solution and to examine the recovered materials by physical or chemical means.

4:1:3:1. Experiments in the Analytical Ultracentrifuge.

Aluminium centrepieces are usually avoided in isopycnic density gradient centrifugation since the concentrated salt solutions which are used have a corrosive effect on metals. The most satisfactory centrepieces are those fabricated from plastics such as Kel-F (Minnesota Mining and Manufacturing Co.) or aluminium filled Epon (Shell Chemical Co.). Cell leaks can be avoided in experiments at high speeds and high densities by tightening the cell slighly more than is usual i.e. to 140 inch-pounds.

Frequently it is necessary to use negative wedge windows in the cells to compensate for the high refractive index generated by the salt solutions (Vinograd and Hearst, 1962).

Density gradient runs in the analytical ultra - centrifuge are usually performed at 20° or 25°, the only reason for this is that much thermodynamic data for the salt solutions have been determined at these temperatures (Vinograd and Hearst, 1962).

4:1:3:2. Experiments in the Preparative Ultracentrifuge.

Preparative isopycnic density gradient centrifugation is ordinarily performed in the high speed swinging-bucket rotors such as the Beckman-Spinco SW 39 and the SW 25. Usually, cellulose nitrate or polyallomer tubes are used

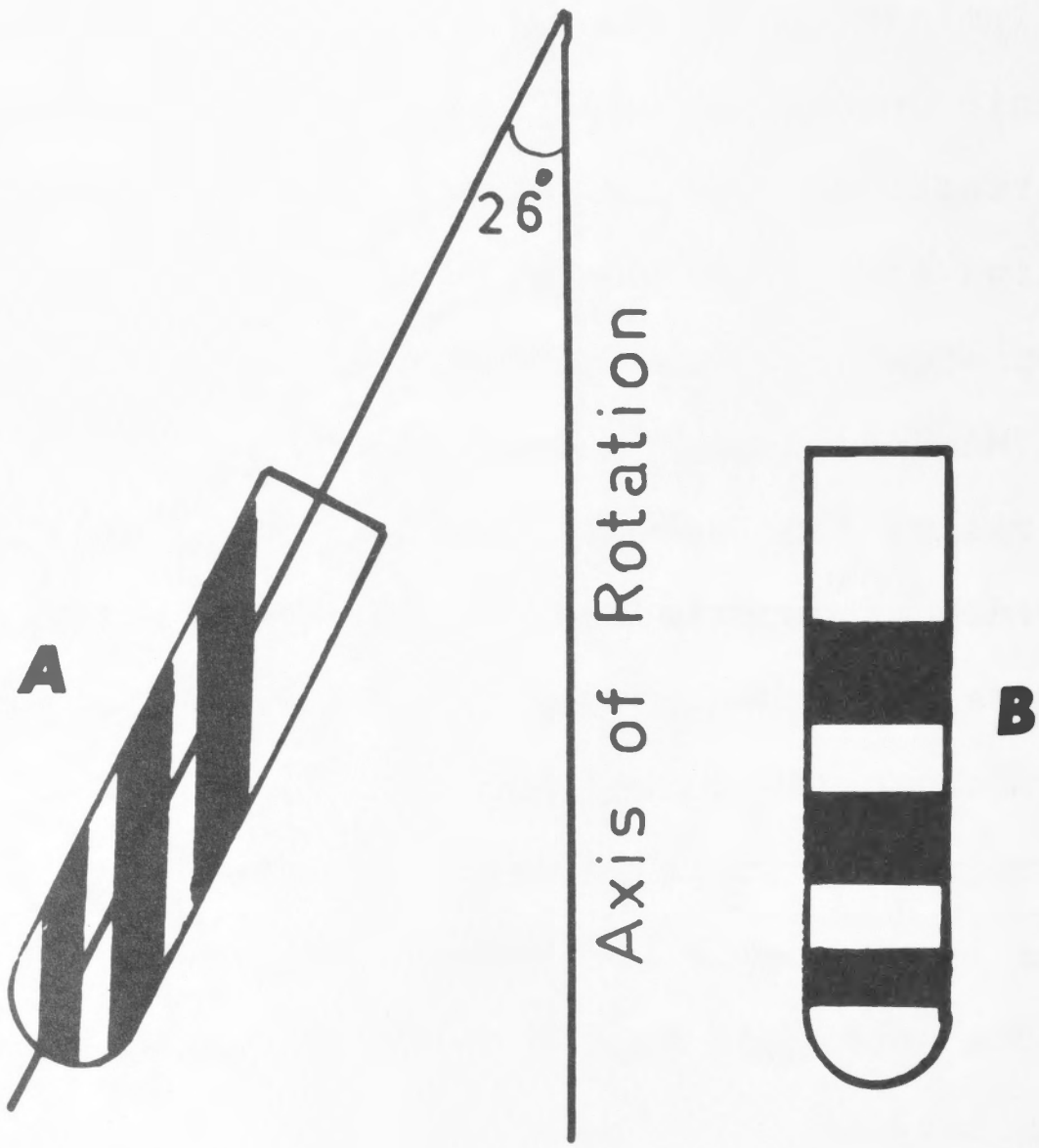


Fig. 4:2. Scale diagram of a tube from the angle head rotor. (A) represents the tube in its speed position (B) the tube in its collecting position. Note the increase of band width upon changing the position of the tube from A to B.

since these allow for easy cutting or perforation when fractionating the material at the completion of centrifugation. If not filled to capacity with the gradient forming solution, a light mineral oil is layered on top of the aqueous solution to prevent tube collapse.

The swinging-bucket rotors have several disadvantages; the SW 39 can attain high angular velocities but can only take small sample volumes, while the SW 25 can accommodate large sample volumes but can be run at low speeds only.

Consideration of such limitations led Fisher, Cline and Anderson (1964) to investigate the use of the angle head rotors for isopycnic density gradient centrifugation. These authors maintain that the basic principle to consider is similar to that applied to hollow cylindrical reorient - ing gradient rotors (Anderson, Price, Fisher, Canning and Burger, 1964), namely there will be a reorientation of the density gradient from the vertical to the horizontal during deceleration; this replaces the mechanical transition of the tubes which occurs in a swinging-bucket rotor.

A change in the physical width of the bands results from the geometry of the tubes and rotor. This is shown diagrammatically in fig. 4:2. However, very little

mixing occurs and there is close agreement between the theoretical and the experimental density gradient (Fisher et al., 1964). Further, Flamm, Bond and Burr (1966) have found that this increase in band width during deceleration is of some advantage, since it results in a greater resolution of material than can be obtained using the swinging-bucket rotors. Pickels (1942) has shown that particles in a tube inclined to the axis of rotation are transported more rapidly due to convection effects associated with the particles accumulated at the cell wall. Because of this and the relatively short sedimentation distance, short equilibrium times are required. This offers a significant advantage over the conventional swinging-bucket rotors.

4:1:3:3. Gradient Forming Materials.

The ideal gradient forming material must be chemically inert to the molecules being studied, capable of covering an adequate density range and easily separable from the macrospecies at the completion of centrifugation. In addition to meeting these requirements certain other criteria must also be considered. As a solute, the gradient material should be sufficiently soluble in aqueous solutions, increase the viscosity of water to only a mild extent and have little effect on the

pH. Finally for preparative centrifugation, the gradient material chould offer no interference with the analytical or physical procedures used for identification.

Sucrose, which is readily available, has been widely used as a gradient forming material; it has several disadvantages. Its solutions are viscous, especially at high concentrations and low temperatures; this results in slower sedimentation. It is difficult to remove completely after centrifugation and thus interfers with carbohydrate analysis (Kundel, Franklin and Muller-Eberhard, 1959). Finally application to isopycnic density gradient centrifugation is limited due to a low density increment. Combination of sucrose with deuterium oxide (Beaufay, Bendall, Baudhuin, Wattiaux and de Duve, 1959) or with thorium oxide (de Duve *et al.*, 1959) have also been reported but again with these materials the density increment is still low. Similarly, organic preparations such as gum arabic, starch, polyvinyl pyrrolidine and Ficoll (Holter and Møller, 1958), Urograffin (Williams, 1966; Schalz, Haslbrunner and Tuppy, 1964) have only limited application because of low density increments.

Meselson *et al.* (1957), Ifft *et al.* (1961), Hearst and Vinograd (1961), Vinograd (1963) and Ludlum and

Warner (1965) have investigated the application of a group of inorganic salts such as CsCl, RbCl, NaBr, $CsSO_4$, Cs acetate and Cs formate as gradient forming materials for isopycnic density gradient centrifugation.

These are highly soluble low molecular weight salts, which have a relatively large density increment and do not effect the viscosity of water to any great extent. Further, being small molecular weight compounds they are easily separable from the macromolecular material at the completion of centrifugation.

Ifft et al. (1961) have investigated the density differences achieved in different gradients of CsCl and have reported that using cells filled to capacity in the SW 39 rotor and centrifuging for 24 hours at 39,000 rpm it is possible to obtain gradients with density differences of up to 0.4 g per cm^3. Further, Hearst and Vinograd (1961) have shown that by using $CsSO_4$ or Cs formate it is possible to obtain densities of the order of 2.0 g per ml, which is far higher than is possible with any of the gradient forming materials mentioned previously.

4:2. APPLICATION OF EQUILIBRIUM GRADIENT CENTRIFUGATION TO PROTEINPOLYSACCHARIDES

4:2:1. METHODS AND MATERIALS.

4:2:1:1. Centrifuges and Rotors.

Preparative experiments were performed in the Beckman-Spinco models L and L2 ultracentrifuges using the swingout SW 39 L and the angle head nos. 30 and 40 rotors. The temperature was maintained at 5°. The rotors were operated at their maximum allowable speed after applying solution density corrections as recommended by the manufacturers, i.e. the maximum rated speed being reduced by the factor

$$(1.2 \text{ g per ml} / \text{density of sample})^{1/2}.$$

Analytical experiments were carried out in the Beckman-Spinco model E ultracentrifuge. The temperature was controlled near 25° with a rotor temperature and indicator control unit. Experiments were performed in the An-D rotor using cells with aluminium filled-Epon double-sector centrepieces. Schlieren optics were used. The speed is indicated in the text.

4:2:1:2. Filling of the Preparative Rotor Tubes.

For the SW 39 rotor, each of the tubes was filled with approximately 5 ml of the appropriate solution under investigation and then they were adjusted to equal weight to ensure correct balance of the rotor.

With angle-head rotors, the tubes were filled at 4°, stored at -20° for 3 hours and then filled to capacity while still cold. This reduced the incidence of tube collapse during centrifugation.

4:2:1:3. Unloading of Tubes.

Rotors were allowed to come to rest without braking. The tubes were removed from the rotor and carefully placed in a vertical position. Fractions were then collected either by the use of a Beckman-Spinco tube slicer or by expelling the contents through a hole in the bottom of the tube by forcing kerosine in through the hole in the tube cap with a micrometer syringe burette.

4:2:1:4. Preparation of Solutions.

The initial investigations of the application of density gradient centrifugation to the separation of aortic proteinpolysaccharides were commenced before the final method of extracting this material from the tissue had been decided, thus, in early sections of this work, the proteinpolysaccharide preparations were similar to those described in section 3:3:2:1, method A while in later stages (especially the work which will be described in chapter 5) the proteinpolysaccharides were extracted from the aorta by the modified method, section 3:3:2:3, method C. The nature of the preparation will be given in the text.

The solutions were prepared for centrifugation by the gravimetric combination of solid CsCl with aqueous solutions of the crude proteinpolysaccharides. A relationship between weight composition and density (Hearst and Vinograd, 1961) was used to compute these combinations i.e.

$$\text{Wt } \% = 137.48 - 138.11\ (1/\rho^{25°})$$

where ρ is the required density. This relationship is valid over the density range 1.2 - 1.9 g per ml (Vinograd, 1963).

4:2:1:5. Density Determination.

These were made using a 0.2 ml constriction pipette as a pycnometer. No attempt was made to control the temperature of the sample during measurements, which were made at room temperature. A value of 0.997 g per ml was used for the density of water under these conditions. Density determinations obtained in this way are accurate to about $\pm$ 0.001 g per ml (Vinograd, 1963).

4:2:1:6. Calculations.

The position of material in the density gradient was established by the chemical analysis of small fractions taken from a sample tube. The position of the fractions were then correlated with radial distance, the radial distance co-ordinate being taken as the mid-point

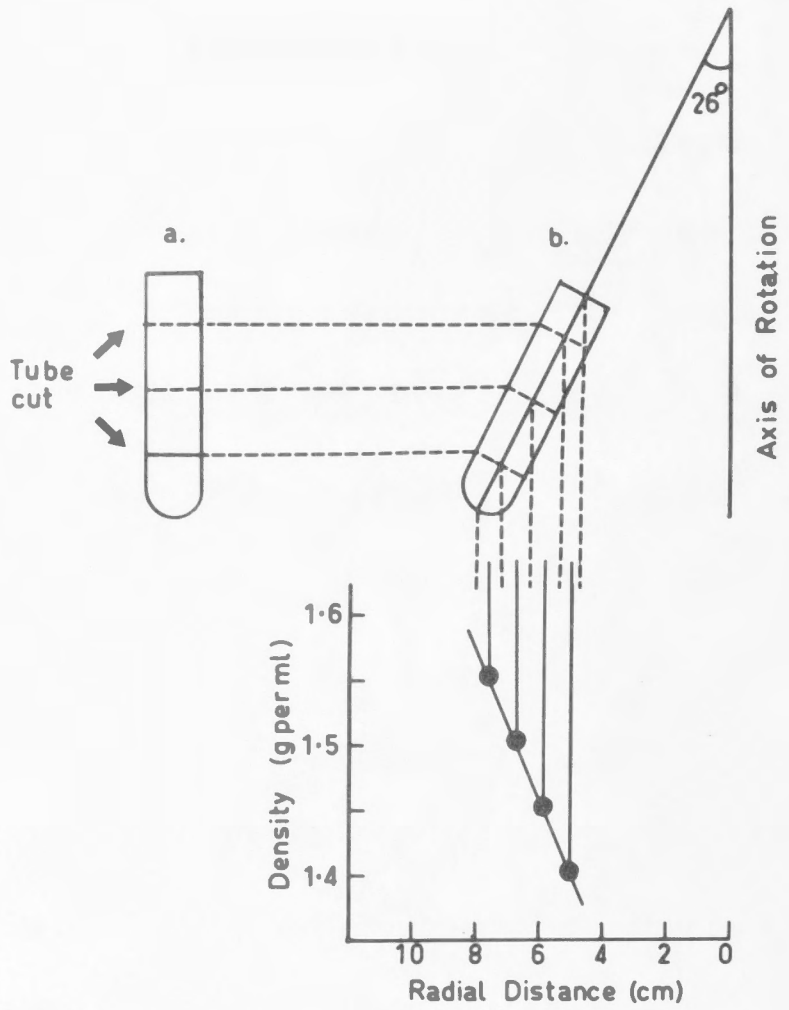


Fig. 4:3. Determination of the position of material within the density gradient for the angle head rotors. Each cut section of tube was measured in sequence (fig 4:3a), this length was then transposed onto a scale diagram of the tube in its speed position (fig 4:3b) which in turn was transposed onto the radial axis. Each section of tube gives two positions on the radial axis, the mid-point of these two positions was taken as the co-ordinate on the graph for plotting the density or analytical quantity in that section of the tube.

of the section of the tube cut with the tube slicer. In the case of the angle head rotors, the radial distance was related by means of a geometrical scale diagram to the section of tube cut. Fig. 4:3 illustrates how this was done.

Each section of tube cut was measured in sequence (fig 4:3a), this length was then transposed onto a scale diagram of the tube in its speed position (fig 4:3b) which in turn was transposed onto the radial axis. Thus each section of tube would give two positions on the radial axis, the mid-point of these two positions was then taken as the co-ordinate on the graph for plotting the density or chemical determinations.

The assumptions made here are not rigorously correct; however since analyses were only performed in order to obtain some idea of the position of the material in the gradient, the assumptions were adequate. Further it will be shown (section 4:2:2:1) that there was close agreement between the theoretical and experimental density gradients.

4:2:2. EXPERIMENTAL INVESTIGATIONS.

4:2:2:1. Theoretical Density Distributions Developed in the Preparative Rotors.

The compositional density gradient can be calculated from the expression for sedimentation equilibrium in a two-component system (Ifft et al., 1961)

$$\beta(\rho)\ \frac{d\rho}{dr} = \omega^2 r \qquad \text{---------(4:1)}$$

where

$$\beta(\rho) = d\ \frac{\ln a}{d\rho} \cdot \frac{RT}{M(1-\bar{v}\rho)} \qquad \text{---------(4:2)}$$

and M, a and $\bar{v}$ are respectively the molecular weight, activity and partial specific volume of the solute; $d\rho/dr$ is the density gradient and ω the angular velocity.

The density ρ, at the point r in the cell is given by

$$\rho = \rho_e + \frac{d\rho}{dr}\ (r - r_e) \qquad \text{---------(4:3)}$$

where r_e is the isoconcentration point i.e. the point at which the density is ρ_e, the initial density of the solution. Combining equations 4:1 and 4:3 we get

$$\rho = \rho_e + \frac{\omega^2 r}{\beta(\rho)}(r - r_e) \qquad \text{---------(4:4)}$$

This equation can be used to compute the relationship between ρ and r provided ρ_e, r_e and $\beta(\rho)$ are known.

These relationships however are only approximate; for equation 4:3 to be valid $d\rho/dr$ must be constant over

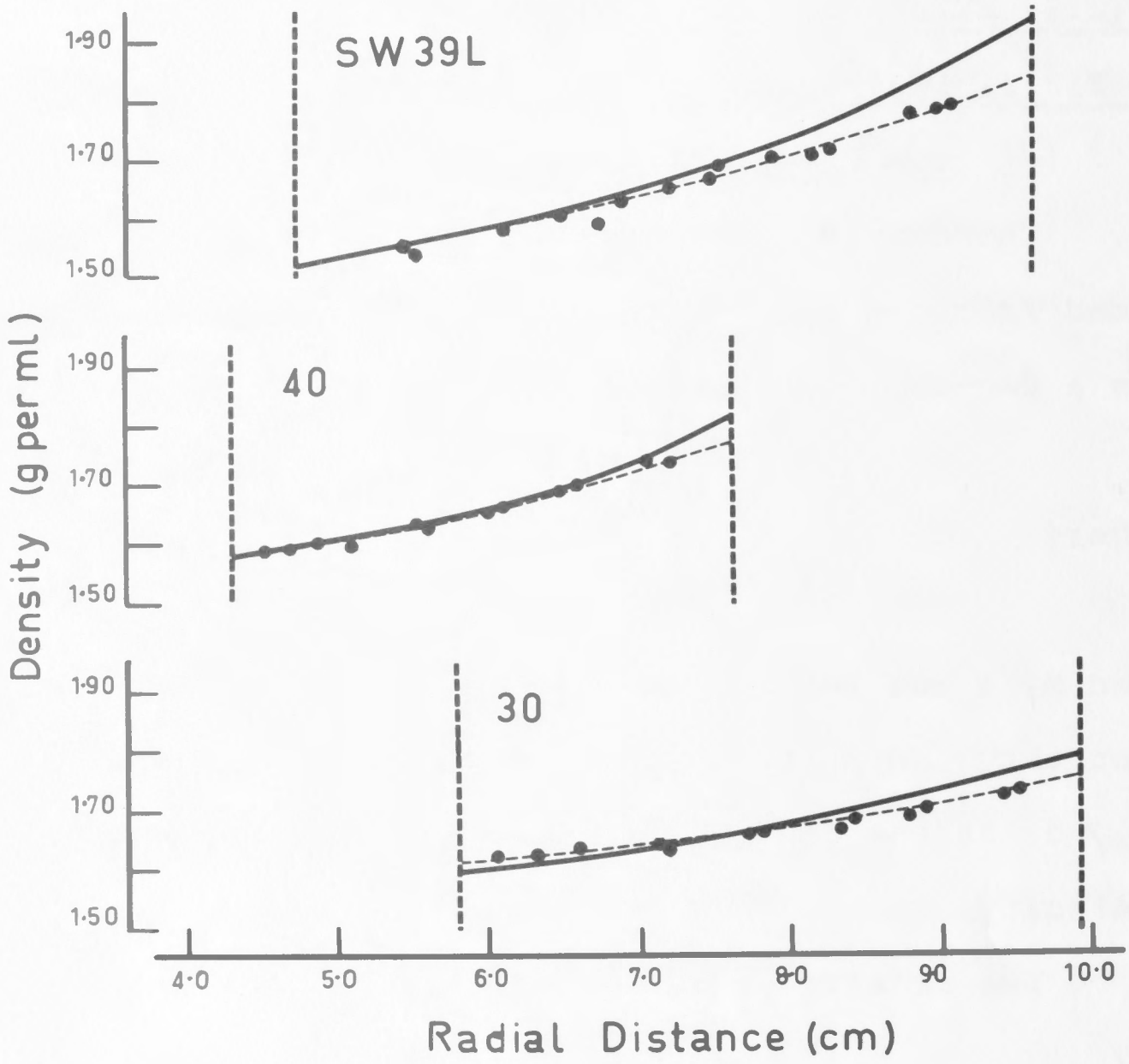


Fig. 4:4. Density distribution in three preparative rotors. Solid line, theoretical distribution, ●- - -● , experimental distribution. CsCl solutions initial density 1.66 g per ml. Tubes filled to capacity were centrifuged at the maximum allowable speed.

the range of $(r - r_e)$ and for equation 4:4 to be valid $\beta(\rho)$ must be constant over the range $(r - r_e)$. Never - theless these approximations are adequate to define the experimental results presented in this work.

Values of $\beta(\rho)$ can be obtained from the data of Ifft et al. (1961). In this work the initial density was always near 1.65 g per ml, hence the value assumed for $\beta(\rho)$ was 1.19×10^9. Assuming that $\beta(\rho)$ is constant the limiting isoconcentration points in sectors (equation 4:5) and in cylinders (equation 4:6) may be estimated

$$r_e = \left| \frac{r_b^2 + r_m^2}{2} \right|^{1/2} \qquad \text{----------(4:5)}$$

$$r_e = \left| \frac{r_b^2 + r_b r_m + r_m^2}{3} \right|^{1/2} \qquad \text{----------(4:6)}$$

where r_m and r_b are the positions of the meniscus and the cell bottom respectively. However in these investigations the position of r_e was assumed to be the midpoint of the tube. The true position of r_e is approximately 0.1 cm nearer the tube bottom in the case of the SW 39 and the no. 40 rotors than the midpoint value. Fig 4:4 compares the theoretical and experimental density distributions developed in three different preparative rotors; the swing-out SW 39 and the angle heads nos. 30 and 40. The initial density in each was 1.66 g per ml. Solutions were centrifuged for 24 hours

at the maximum allowable speed of the rotor. The decrepancy between the theoretical and experimental distributions observed near the bottom of the cell has been attributed to back diffusion (Ifft et al., 1961). In table 4:1 the theoretical and experimental gradients are compared. These values do not represent true gradients but simply the extremes in density values with respect to radial distance.

Table 4:1. The Theoretical and Experimental Density Gradients Developed in Preparative Ultra-centrifuge Rotors. (The initial density of the solutions was 1.66 g per ml).

ROTOR	$10^{-6} \times \omega^2 r_e$	DENSITY GRADIENTS (g per cm^4)	
		Theoretical	Experimental
SW 39 L	95.7	0.08	0.06
40	79.3	0.07	0.06
30	55.1	0.05	0.03

4:3:2:2. The Conditions of Equilibrium.

The time t required for the concentration of a single species to be within one percent of its equil-ibrium value every-where between the centre of the Gaussian distribution and $\pm$ two standard deviations may be estimated (Meselson et al., 1957) from the relation

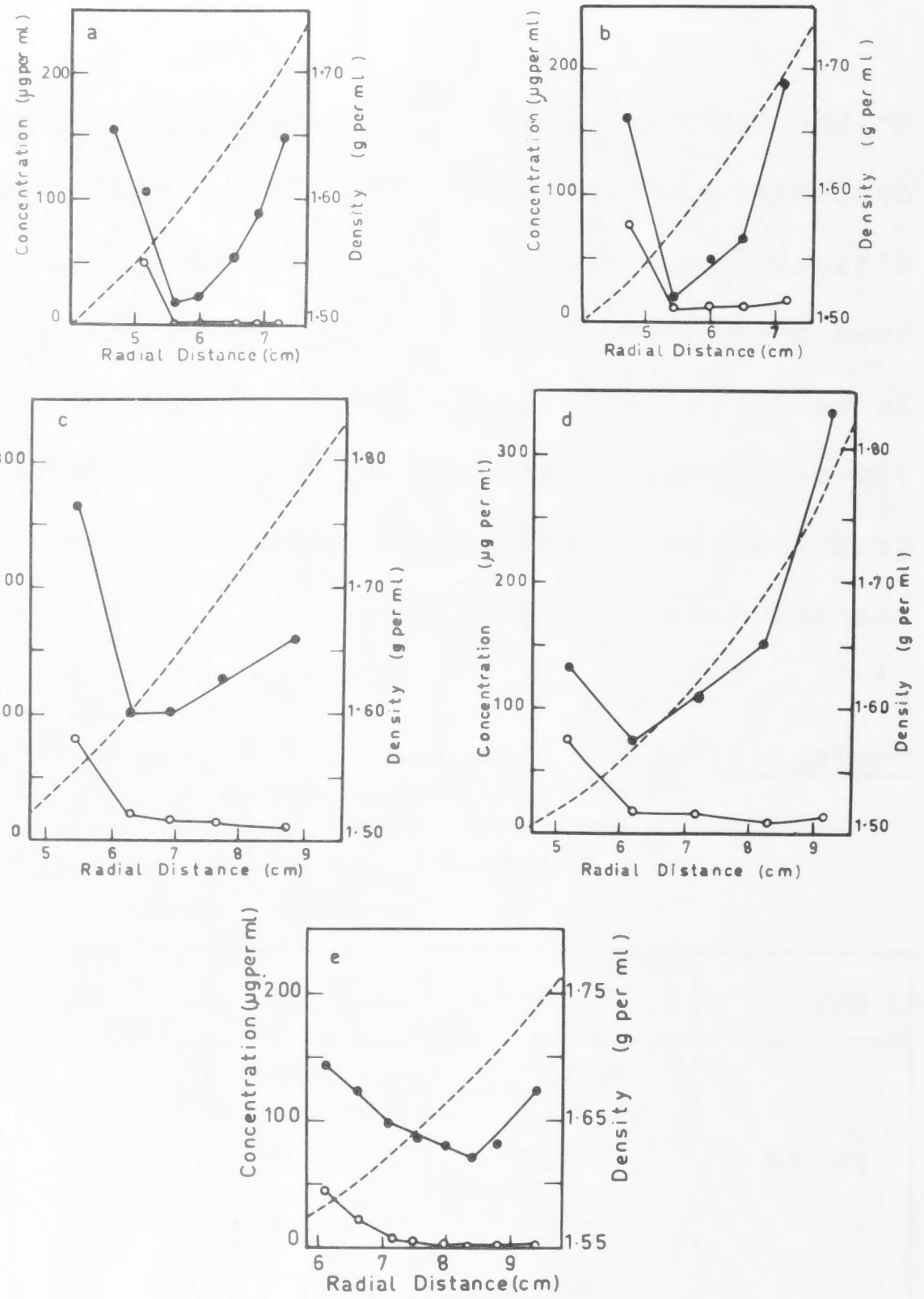


Fig. 4:5. Distribution of uronic acid and sialic acid after centrifuging a mixture of proteinpoly-saccharides from aorta in a CsCl gradient of initial density 1.65 g per ml. (a) 40 rotor after 48 hours, (b) 40 rotor after 100 hours, (c) SW 39 rotor after 48 hours, (d) SW 39 rotor after 100 hours and (e) 30 rotor after 48 hours.
●——● , uronic acid; ○——○ , sialic acid;
- - - - - , density gradient.

$$t = \frac{\sigma^2}{D}\left(\ln\frac{L}{\sigma} + 1.26\right) \quad L > \sigma$$

where σ is the standard deviation of the equilibrium distribution, D the diffusion coefficient of the macrospecies and L is the length of the liquid column in which the macrospecies are uniformly distributed at the beginning of centrifugation. The treatment assumes that the equilibrium density gradient is fully established at zero time. Estimates of the times required to reach a near approach to equilibrium suggest periods of about 100 hours ought to be satisfactory for typical connective tissue proteinpolysaccharides.

To determine experimentally the time required for equilibrium to be reached when a solution of protein - polysaccharides is centrifuged in a density gradient, a crude solution of aortic proteinpolysaccharides (prepared as described in section 3:3:3:1, method A) was adjusted to an initial density of 1.65 g per ml and centrifuged in the SW 39 and 40 rotors for 48 and 100 hours and in the No. 30 rotor for 48 hours. Fig 4:5 illustrates the results of these experiments.

The similarity between the 48 and the 100 hour distributions for the No. 40 rotor suggested that the system was close to equilibrium after 48 hours. However, in the case of the SW 39 swing-out rotor, the chemical distribution indicated that the time required for a near approach to equilibrium would be of the order of 72 to 100 hours.

These data suggested that the No. 40 rotor would be the most satisfactory rotor to use for the separation of these proteinpolysaccharides because it has, compared with the SW 39 rotor, a relatively large sample volume capacity and equilibrium is approached faster. No advantage could be seen for using the No. 30 rotor because, although its sample volume capacity is large the gradient established is significantly smaller than the other two rotors.

4:2:2:3. Stability of the Gradient after Deceleration.

The time required to fractionate a number of tubes at the completion of centrifugation may vary from one-half to two hours. The following experiment was designed to see if any significant differences could be observed in the dis - tribution of material when a sample tube had been left standing in a vertical position at room temperature for at least one hour after deceleration.

A solution of proteinpolysaccharide material from aorta (prepared as in section 3:3:2:1 method A) was adjusted to an initial density of 1.63 g per ml with CsCl and centrifuged in the No. 40 rotor for 48 hours in the model L ultracentrifuge. After this time and two tubes were carefully removed from the rotor and one tube was fractionated immediately. The other tube was allowed to stand for one hour at room temperature before being fractionated. The density and the uronic acid content of each of the fraction

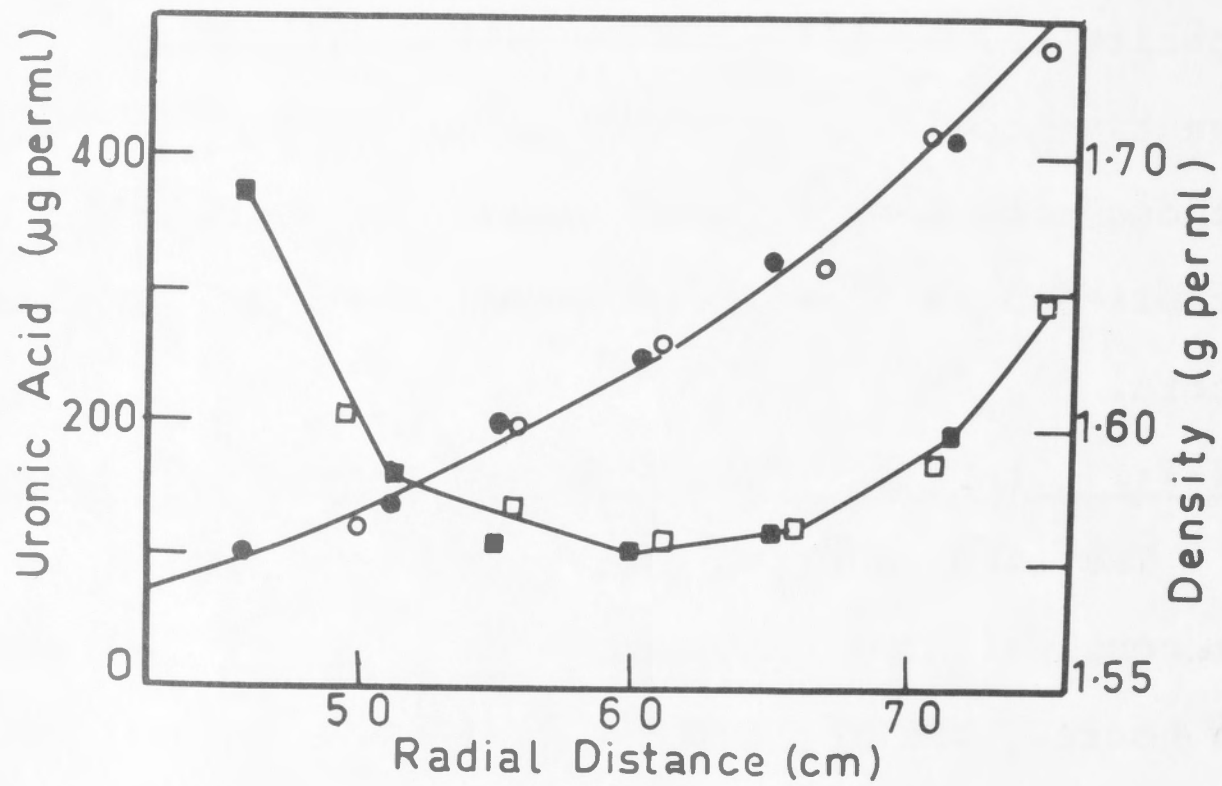


Fig.4:6. Comparison of the uronic acid and density distributions between two sample tubes; one fractionated immediately after the rotor has come to rest,(open symbols), and the other after an interval of one hour, (shaded symbols). The density is shown by ○ ● and the uronic acid by □ ■ .

was determined. The results are illustrated in fig. 4:6. There is close agreement between the density and uronic acid determinations for both tubes, indicating that there has been little disturbance to the gradient or to the material within the gradient during this time.

4:2:2:4. Separation of Fetuin and a Glycosaminoglycuronoglycan Protein.

Having established the optimal conditions by which a near approach to equilibrium might be obtained, but before applying the method to the separation of the proteinpolysaccharides present in aorta extract, a separation of two well-characterized compounds fetuin (Graham, 1961) and a chondroitin sulphate protein (Buddecke, 1963) were examined. The buoyant densities of these two materials approximate respectively to the values expected for the glycoprotein (Radhamrishnamurthy et al., 1964) and the chondroitin sulphate protein (Buddecke et al., 1963) components of an aorta extract.

This separation was performed in the SW 39 rotor. Solution 1 contained fetuin only, solution 2, the chondroitin sulphate protein only and solution 3, a mixture of the two. The solutions were all adjusted to an initial density of 1.63 g per ml and centrifuged for

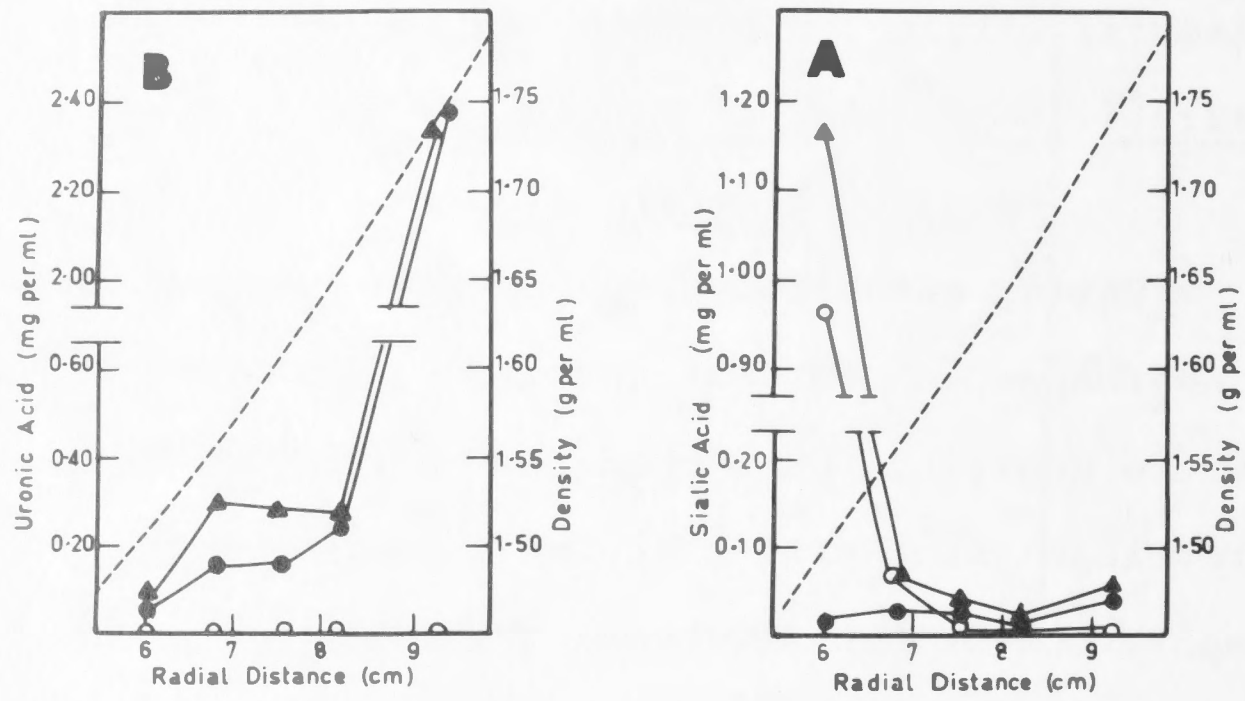


Fig. 4:7. Distribution of uronic acid and sialic acid after centrifuging samples of fetuin (15 mg), chondroitin sulphate protein (15 mg) and fetuin (15 mg) plus chondroitin sulphate protein (15 mg) for 72 hours in a CsCl gradient in the SW 39 rotor. The initial density was 1.63 g per ml. The density gradient is shown by the line - - - - , ●——● , chondroitin sulphate protein; O——O , fetuin; ▲——▲ , fetuin plus chondroitin sulphate protein. (A) distribution of sialic acid, (B) distribution of uronic acid.

72 hours at 35,000 rpm. The results are shown in fig 4:7. It is clear that the separation of these two materials by density gradient centrifugation is complete.

4:3. SEPARATION OF THE PROTEINPOLY-SACCHARIDES FROM AN AORTA EXTRACT.

A solution of proteinpolysaccharides prepared as described in section 3:3:3:1, method A was adjusted to an initial density of 1.63 g per ml and centrifuged for 48 hours in the no. 40 rotor. After this time the material in the tubes was separated into several fractions and the identical fractions from two tubes were pooled. Chemical analysis was performed on these pooled fractions. A typical distribution of material within the gradient is shown in fig 4:8.

The accumulation of sialic acid-containing material near the meniscus, of uronic acid-containing material near the bottom and near the meniscus, of glucosamine near the meniscus and of galactosamine near the bottom of the tube are clearly evident. Material near the centre of the tube is characterized by a high absorbance at 260 mμ and is presumably nucleic acid. This fraction contains only small amounts of hexosamine, uronic acid

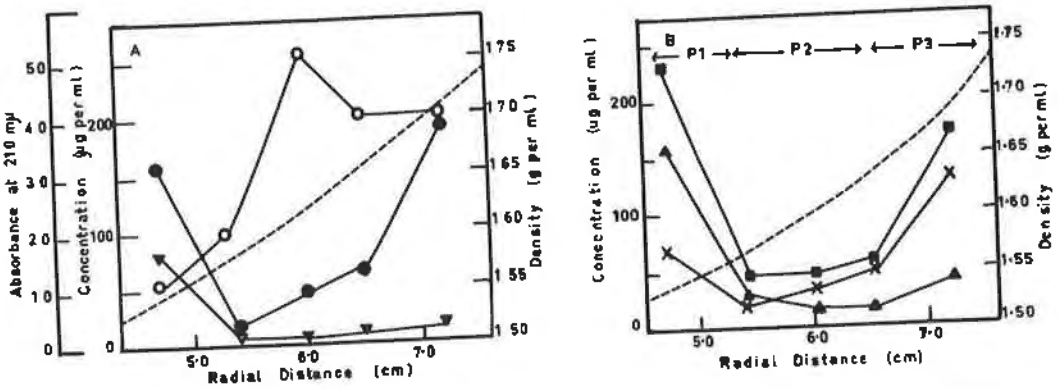


Fig.4:8. Distribution of material after centrifuging a solution of proteinpolysaccharide from aorta to equilibrium in a CsCl gradient, initial density 1.65 g per ml. in the no. 40 rotor. Details of the preparation are given in the text. The density is shown by the line,- - - -. Contents of the fractions obtained by tube slicing were pooled as shown between the arrows to give the fractions PI, P2, P3. (a) 0——0, absorbance at 210 mμ; ●——●, uronic acid; ▼——▼, sialic acid; (b) ■——■ total hexosamine; ▲——▲, glucosamine; X——X, galactosamine.

and sialic acid. A thin gel layer was always present at the meniscus. It was easily removed from the tube in one piece and was difficultly soluble in water. The gel contained variable amounts of uronic acid, hexosamine and sialic acid and in view of its low density, is assumed to be largely protein. Material immediately below the meniscus exhibited higher extinction values at 210 mμ than the material near the cell bottom (table 4:2) indicating higher protein concentrations in the regions of lower density.

In the light of these analyses a preparative experiment was carried out using the no. 40 rotor. Fractions obtained from each of ten tubes were pooled as indicated in fig 4:8. After dialysing free of CsCl they were concentrated by ultrafiltration. An attempt was then made to identify these three fractions by chemical analysis and by precipitation onto cellulose with cetylpyridinium chloride followed by elution with increasing concentrations of $MgCl_2$. The results of these analyses are shown in tables 4:2 and 4:3.

Table 4:2. Chemical Composition of the Fractions obtained from the Preparative Density Gradient Centrifugation of the Proteinpolysaccharides from Aorta Extract.
Fractions from ten centrifuge tubes were pooled as indicated in fig 4:8. All figures are the mean values of replicate determinations and are quoted in μmoles per ml.

ANALYSIS	FRACTION		
	P1	P2	P3
Uronic Acid	0.65	0.26	0.70
Sialic Acid	0.26	0.09	0.06
Galactosamine	0.30	0.14	0.52
Glucosamine	0.69	0.08	0.16
Sulphate	0.10	0.13	0.53
Glucuronic acid: Iduronic acid	approx.2	approx.1	approx.6
Absorbance 210 mμ	13.3	6.1	5.8

The results of the analyses shown in table 4:2 show that fraction P1 contains glycosaminoglycan protein and glycosaminoglycuronoglycan protein. This is indicated by the presence of almost all the sialic acid, the relative large excess of hexosamine over uronic acid (some allowance for the colour yield of L-iduronic acid in the carbazole reaction being made) and the

predominance of glucosamine over galactosamine. If there is no galactosamine in the glycosaminoglycan fraction (Radhakrishnamurthy et al., 1964)and if the uronic acid-containing materials of aorta contain hexosamine and uronic acid in approximately equimolar amounts (Antonopoulos et al., 1965)it follows that the glycosaminoglycuronoglycan fraction contains both glucosamine and galactosamine. Hence, this fraction contains besides glycosaminoglycan at least two glycosaminoglycuronoglycans. By making allowance for the low colour yield of iduronic acid in the carbazole reaction and using the estimated amount of iduronic acid in this preparation (Table 4:2), iduronic acid and galactosamine are found to be present in near equimolar amounts. This suggests that one of the glycosaminoglycuronoglycans is dermatan sulphate. However, the low sulphate value (approximately half of the expected value) would not support this contention. The presence of glucuronic acid and glucosamine together with the low sulphate value would suggest that the other glycosaminoglycuronoglycan is hyaluronic acid.

Fraction P3 contains close to equimolar amounts of hexosamine and uronic acid (no allowance for the low colour yield of iduronic acid in the carbazole reaction

has been made here owing to the relatively small amount present). From this we conclude that this fraction contains only complexes of glycosaminoglycuronoglycans which also contains some small amount of sialic acid. Approximately 75 percent of the hexosamine is galactosamine, the remainder being glucosamine. If the glucosamine was attributed to a heparan sulphate-like protein-polysaccharide similar to that described by Muir (1961) and Jacobs and Muir (1963), then this would account for about 0.1 μmole per ml of sulphate and 0.2 μmole per ml of uronic acid, leaving 0.5 μmole per ml uronic acid, 0.52 μmole per ml hexosamine and 0.43 μmole per ml sulphate. This remaining material would be consistent with the presence of a chondroitin sulphate probably chondroitin 6-sulphate (Buddecke and coworkers, 1961, 1963). The small amount of iduronic acid present in this fraction could be attributed to heparan sulphate (Cifonelli and Dorfman, 1962).

The apparent densities of glycosaminoglycuronoglycans present in aortic wall, calculated from partial specific volumes are: dermatan sulphate (6 percent peptide), 1.75 g per ml (Tanford et al., 1964), but material with about 30 percent protein would probably have a density nearer 1.65 g per ml and if, as may be

the case (Table 4:2) this material was partially desulphated, an even lower density might result; chondroitin 6-sulphate (20 percent peptide), 1.82 g per ml, (Mathews and Lozaityte, 1958); hyaluronic acid (thought to be protein free) 1.66 g per ml (Silpananta et al., 1967) but a complex containing about 20 percent protein would probably have a density of about 1.55 g per ml; heparan sulphate would probably have a density of the order of 1.7 to 1.8 g per ml and a somewhat lower value if combined with protein. These data are consistent with the suggestions made above concerning the composition of fraction P1 and P3.

The fractionations on cellulose with cetylpyridinium chloride (Table 4:3) were only partly successful. With fraction P1 only about half the uronic acid, sialic acid and hexosamine could be recovered from the column. The recovered material was distributed between four fractions which were eluted respectively with 1% cetylpyridinium chloride, 0.3M NaCl 0.5M $MgCl_2$ and 0.75M $MgCl_2$. The initial fraction contained uronic acid, sialic acid and hexosamine. The last three fractions contained uronic acid and hexosamine. The analysis of fraction P3 is given in Table 4:3. Recoveries of about 90 percent were obtained. The material not retained by the column (1% cetylpyridinium chloride fraction) contained uronic acid, sialic acid and hexosamine.

Table 4:3. Analysis of Fractions P1 and P3 by Precipitation onto Cellulose with Cetylpyridinium Chloride. The fractionation was carried out using the batch method (Buddecke et al., 1963). The results are expressed as mg of material contained in each fraction.

FRACTION P1.

ELUTING SOLVENT	URONIC ACID	HEXOSAMINE	SIALIC ACID
1 % cetylpyridinium chloride	145	185	93
0.3M NaCl	175	198	0
0.3M $MgCl_2$	0	0	0
0.5M $MgCl_2$	103	50	0
0.75M $MgCl_2$	108	102	0
Initial Material	726	967	363
Material recovered	531	535	93

FRACTION P3.

ELUTING SOLVENT	URONIC ACID	HEXOSAMINE	SIALIC ACID
1 % cetylpyridinium chloride	900	708	160
0.3M NaCl	55	0	0
0.3M $MgCl_2$	74	0	0
0.5M $MgCl_2$	195	107	0
0.75M $MgCl_2$	531	522	39
Initial Material	1595	1420	199
Material recovered	1455	1337	230

Most of the remaining material which contains predominantly uronic acid and hexosamine was eluted with 0.75M $MgCl_2$.

The partial failure of the method would appear to be due to incomplete precipitation of the glycosaminoglycuronoglycan fractions by the cetylpyridinium chloride. It is known that homogeneous protein polysaccharide fractions are not necessarily obtained as a result of a single chromatography as used in these experiments and it is possible that a more complete resolution of the fractions P1 and P3 might be obtained by rechromatography. This possibility has not been investigated. However, the results of the partial separation do indicate the presence of a hyaluronic acid (0.3M NaCl) and a chondroitin sulphate (0.75M $MgCl_2$) fraction in fraction P1, and of a chondroitin sulphate (0.75M $MgCl_2$) fraction in fraction P3.

In view of these findings a more detailed chemical analysis of fractions P1 and P3 was made. The results are shown in table 4:4.

These results confirm the earlier findings that fraction P1 contains glycoprotein and glycosaminoglycuronoglycan protein and P3 contains only glycosaminoglycuronoglycan protein.

Table 4:4. Chemical Composition of the Fractions P1 and P3
Details of the preparation are given in the text.

ANALYSIS (mg per 100 mg dry weight)	P1	P3
Glucuronic acid	11.79	17.50
Hexosamine	11.23	13.69
Glucosamine	7.88	2.39
Galactosamine	3.35	11.31
Sialic acid	5.15	3.25
Hexose[a]	11.26	18.84
Sulphate[b]	2.22	7.20
Protein[c]	56.95	12.56
Protein[d]	45.82	8.40
Molar ratio. Hexosamine = 1.00		
Uronic acid	0.97	1.18
Hexosamine	1.00	1.00
Glucosamine	0.70	0.17
Galactosamine	0.30	0.82
Sialic acid	0.26	0.14
Hexose	0.25	1.37
Sulphate	0.37	0.98
Amino acids (μmoles per 100 μmoles amino acid determined).		
Lys	7.8	3.6
His	1.9	1.7
Arg	4.7	2.3
Asp	10.4	8.7
Thr	6.6	8.8
Ser	6.8	9.7
Glu	15.5	14.8
Pro	5.6	7.0
Gly	7.2	17.5
Ala	8.5	7.8
CyS	1.3	trace
Val	5.8	5.9
Met	1.1	trace
Ile	3.3	3.3
Leu	7.2	6.2
Tyr	2.8	0.5
Phe	3.3	2.2

[a], as galactose; [b], as SO_4^{2-}; [c], determined by the method of Lowry *et al.* (1951); [d], calculated from the amino acid analysis.

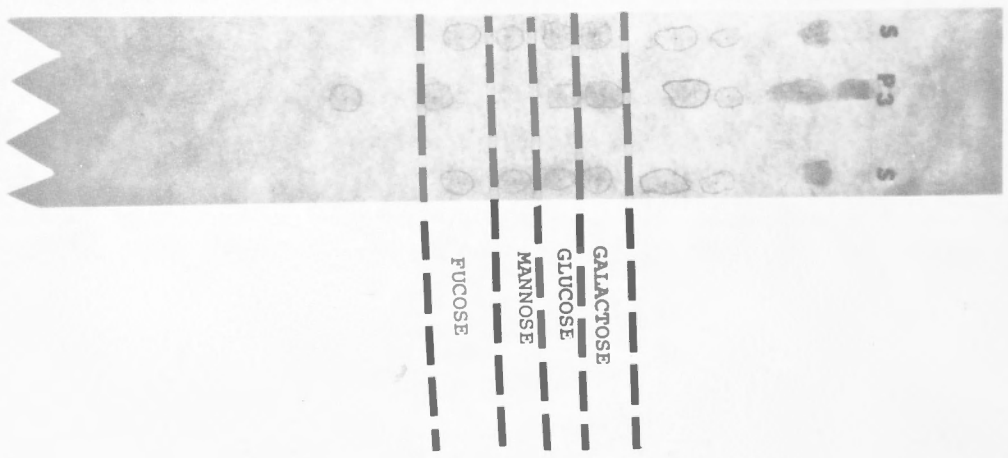


Fig. 4:9. Paper chromatography of fraction P3. Hydrolysed 7 hours in 2N HCl at 100°. Solvent - Butanol : Pyridine : water (6:4:3). Stain - Aniline hydrogen phthalate.

Fraction P1 contains approximately 50 percent protein, 13 percent uronic acid and 20 percent hexosamine. From the molar ratios of the various constituents it can be seen that the ratio of sulphate is approximately equal to that of galactosamine. If it is assumed that hyaluronic acid protein is present then the sulphate would be associated mainly with the second glycosaminoglycuronoglycan moiety i.e. dermatan sulphate.

The surprising feature of the chemical analysis of fraction P3 is the high hexose content which is present in similar amounts to uronic acid. Paper chromatography of this fraction revealed that the hexoses present were galactose, glucose and probably fucose (fig 4:9). This could indicate the presence in aorta of a hitherto unrecognized carbohydrate species.

The amino acid profiles of these two fractions show that the Fraction P1 is the more basic. This could mean the presence of a protein or glycoprotein that is basic in nature similar to that envisaged by Meyer (1966a,b). The only other significant difference is the high content of glycine in Fraction P3.

Concluding Remarks.

The aim of this investigation was to see whether or not the technique of isopycnic density gradient centri - fugation could be applied to the separation of protein-polysaccharides especially those contained in aqueous extracts of aorta.

The method proved completely successful in the separation of the model system containing fetuin and a chondroitin sulphate protein. When applied to the more complex mixture present in aqueous extract of aorta the method effected a partial separation only. Of the main fractions obtained (P1 and P3) analytical results have shown that fraction P1 contains glycoprotein and glycosaminoglycuronoglycan protein while fraction P3 contains only glycosaminoglycuronoglycan proteins.

It was mentioned in the previous chapter that some of the methods of separation and purification cited might be applicable to the separation of the proteinpoly-saccharides present in aorta if a successful initial separation could be made. The technique of density gradient centrifugation has achieved this. Thus methods of further separating these two fractions could be attempted. Some results are presented in chapter 5.

Chapter 5.

Examination of Fractions P1 and P3.

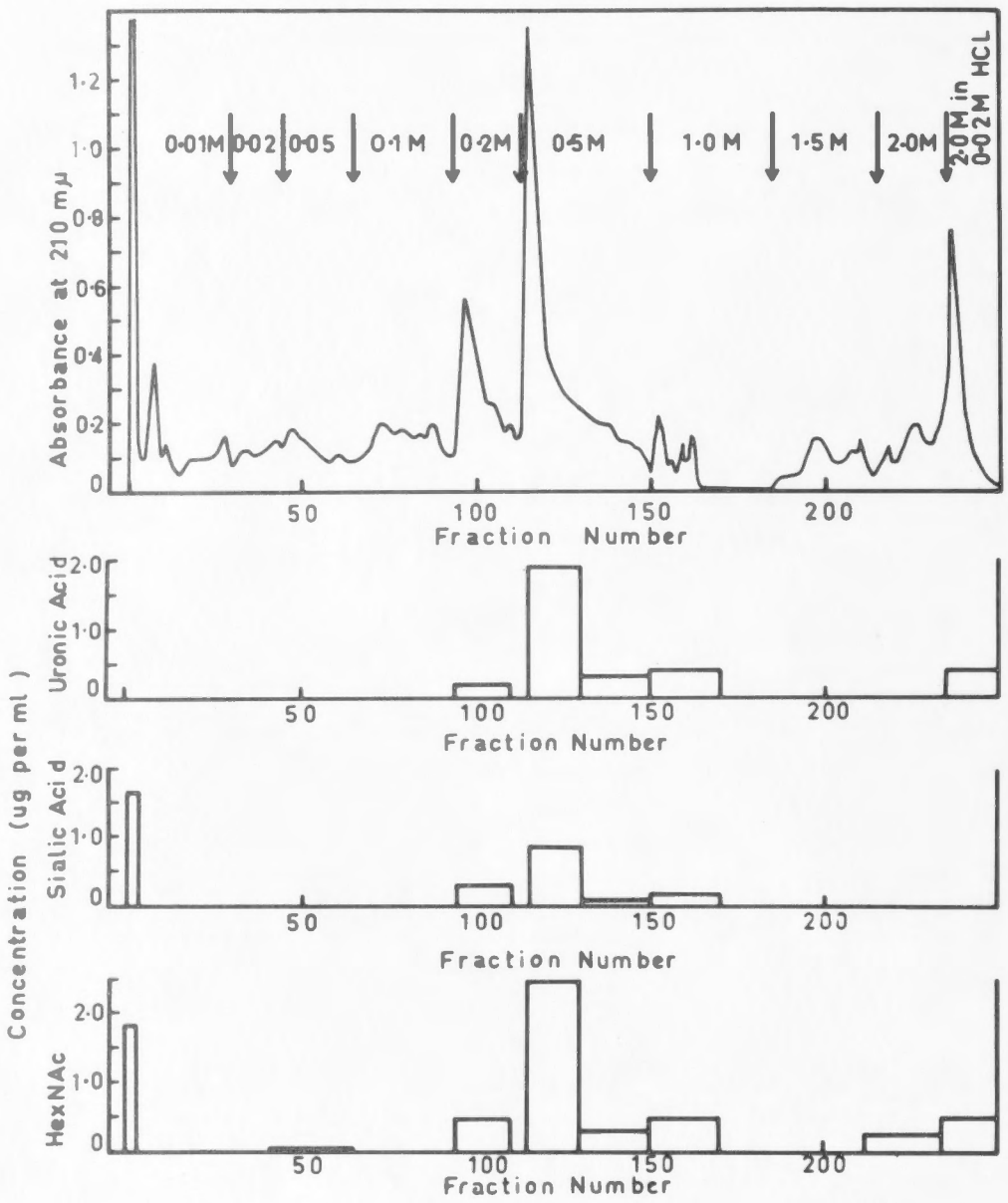


Fig. 5:1. Chromatography of fraction P1 on DEAE-cellulose. Details of the preparation are given in the text. 8.0 mg of material in 5.0 ml of solution was applied to the column (18.0 x 1.04 cm). Gradient elution was carried out discontinuously, the changes in composition of the eluting solvent are indicated by arrows. The elution pattern was determined by measuring the extinction of each fraction (10 ml) at 210 mμ. The chemical analysis of the pooled fractions are illustrated as histograms.

5:1. EXAMINATION OF FRACTION P1.

Fraction P1 was obtained from the low density region (1.50 - 1.58 g per ml) after a crude extract of porcine aorta was centrifuged to equilibrium in a CsCl density gradient of initial density 1.63 g per ml (section 4:3).

5:1:1. COLUMN CHROMATOGRAPHY ON DEAE-CELLULOSE.

Column chromatography on DEAE-cellulose has been frequently used for the isolation and purification of glycoprotein material (see section 3:2:3:2). This method was therefore investigated in an attempt to isolate the glycoprotein material of fraction P1.

A solution of fraction P1 (8 mg in 5.0 ml) was applied to a DEAE-cellulose column (18.0 x 1.04 cm), previous equilibrated with 0.01M NaCl. Material was eluted from the column by means of a discontinuous gradient of NaCl and finally with acidified NaCl. The flow rate was 23.5 ml per sq cm per hour and 10 ml fractions were collected. The elution pattern determined by measuring the extinction of each fraction at 210mμ is shown in fig. 5:1.

Fractions comprising definite peaks were pooled as also were the intermediate fractions. After dialysing free of NaCl and concentrating by ultrafiltration to a known volume, chemical analyses were performed on each of the pooled fractions. The results are included in fig 5:1.

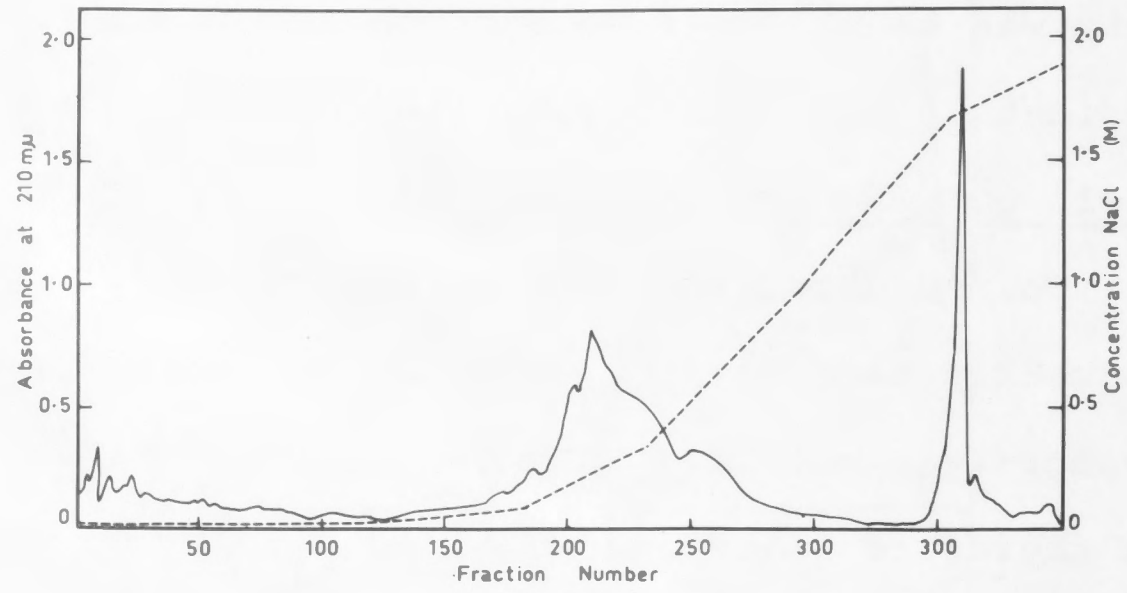


Fig. 5:2. Chromatography of fraction P1 on DEAE-cellulose. Details of the preparation are given in the text. 25 mg of material in 10 ml of solution was applied to column (40.0 x 1.5 cm). Continuous gradient elution is indicated by the line - - - -. The elution pattern was determined by measuring the extinction of each fraction (10 ml) at 210 mμ. Fractions 200 - 250 were pooled, dialysed against distilled water, concentrated by ultrafiltration and reapplied to DEAE-cellulose column (fig. 5:3).

The actual measured values of the analyses are given in appendix 1.

It is evident that three major fractions are present. The first eluted with 0.01N NaCl contains sialic acid and hexosamine but no uronic acid. Material comprising the second fraction contains uronic acid, sialic acid and hexosamine, while the final fraction eluted with acidified NaCl contains only uronic acid and hexosamine. Consideration of this elution pattern suggested that an improved resolution might be possible if a continuous rather than a discontinuous gradient was used.

A solution of fraction P1 (25 mg in 10 ml) was applied to a larger column (40.0 x 1.5 cm) of DEAE-cellulose. Material was eluted by means of a continuous NaCl gradient and finally with acidified NaCl. The flow rate was 11.3 ml per sq cm per hour and 10 ml fractions were collected. The elution pattern determined as before is illustrated in fig. 5:2.

No advantage appears to have been gained by using such a gradient, the resolution of material, especially that comprising the middle substance (i.e. fractions 200-250)is inferior to that obtained in the previous experiment.

In an attempt to obtain a better resolution of the material comprising fractions 200-250, these several fractions were pooled, dialysed free of NaCl, concentrated

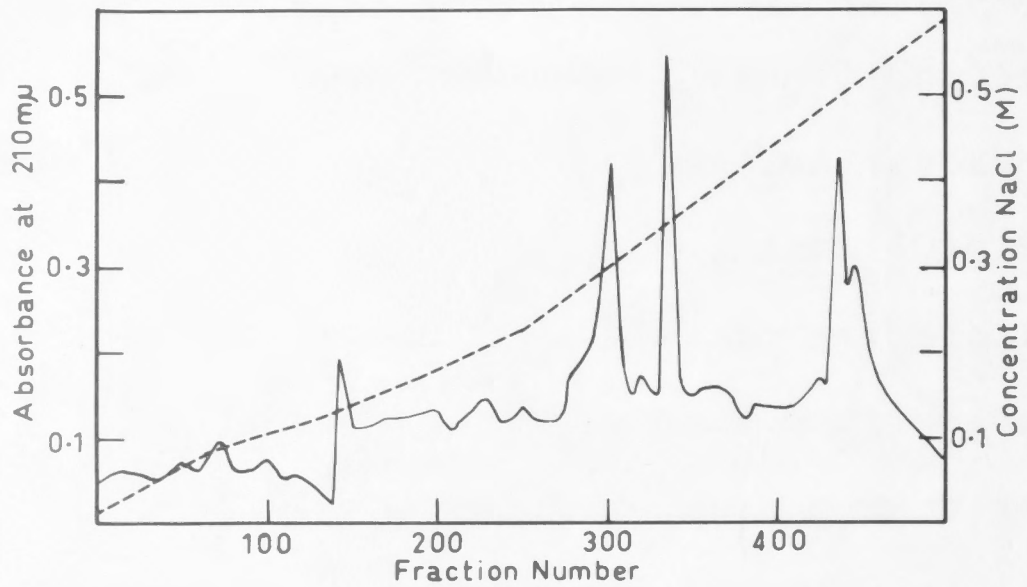


Fig.5:3. Rechromatography of the pooled fractions 200 - 250 (fig 5:2) on DEAE-cellulose. Continuous gradient elution is indicated by the line - - - -. The elution pattern was determined by measuring the extinction of each fraction (10 ml) at 210 mμ.

and again applied to a DEAE-cellulose column.

The pooled fractions, (3.7 ml containing 4 mg of material) were eluted from the column (18.0 x 1.04 cm) with a continuous NaCl gradient. The flow rate was 23.5 ml per sq cm per hour. The elution pattern determined as previously described is shown in fig 5.3. No clear-cut separation has been obtained.

The results of this and the previous experiments demonstrated that although chromatography on DEAE-cellulose did effect a partition of the fraction P1 into a number of fractions, it was obvious that no single discrete fraction was present in these preparations. Indeed the results suggest a family of compounds each with slightly different charge so that a continuous elution of material occurred. The investigation of fraction P1 by this method was therefore discontinued.

5:1:2. EXAMINATION OF FRACTION P1 IN A DENSITY GRADIENT.

The fraction P1 contains glycoprotein, glycosamino-glycuronoglycan protein and probably some non-specific protein. Thus it should be feasible to further fraction-ate this material by recentrifuging in a CsCl gradient that has an initial density near the density range where this material was obtained initially i.e. 1.54 g per ml.

Fig. 5:4. Density gradient sedimentation equilibrium of fraction P1. Details of the preparation are given in the text. The initial density was 1.493 g per ml. The phase plate angle was 60°. (A) 21.7 hours after reaching a speed of 44,770 rpm, (B) 12.6 hours after reducing speed to 29,500 rpm.

5:1:2:1. Analytical Gradient Centrifugation of Fraction P1.

An aliquot of fraction P1 adjusted to an initial density of 1.493 g per ml with solid CsCl was centrifuged to equilibrium at 44,770 rpm in the analytical ultracent - rifuge. The resulting density extended from 1.427 g per ml at the meniscus to 1.564 g per ml at the cell bottom. Fig 5:4 A illustrates the presence in fraction P1 of components with densities near 1.43 g per ml and near 1.56 g per ml, respectively. The greater proportion of material appears to be in the lower density region. By slowing the rotor to 29,500 rpm (density 1.464 - 1.524 g per ml), accumulation of material near the meniscus and near the cell bottom was more obvious (fig 5:4 B). Such results could indicate only density heterogeneity. Further investigations of the fractionation by density difference were therefore made by centrifuging in CsCl solution in the preparative ultracentrifuge.

5:1:2:2. Preparative Gradient Centrifugation of Fraction P1.

A solution of fraction P1 adjusted to an initial density of 1.5 g per ml with CsCl was centrifuged at 35,000 rpm for 48 hours in the model L ultracentrifuge (no. 40 rotor). After this time a sample tube was cut into several fractions and the position of the material within the gradient determined from chemical analysis. The results are shown in fig 5:5.

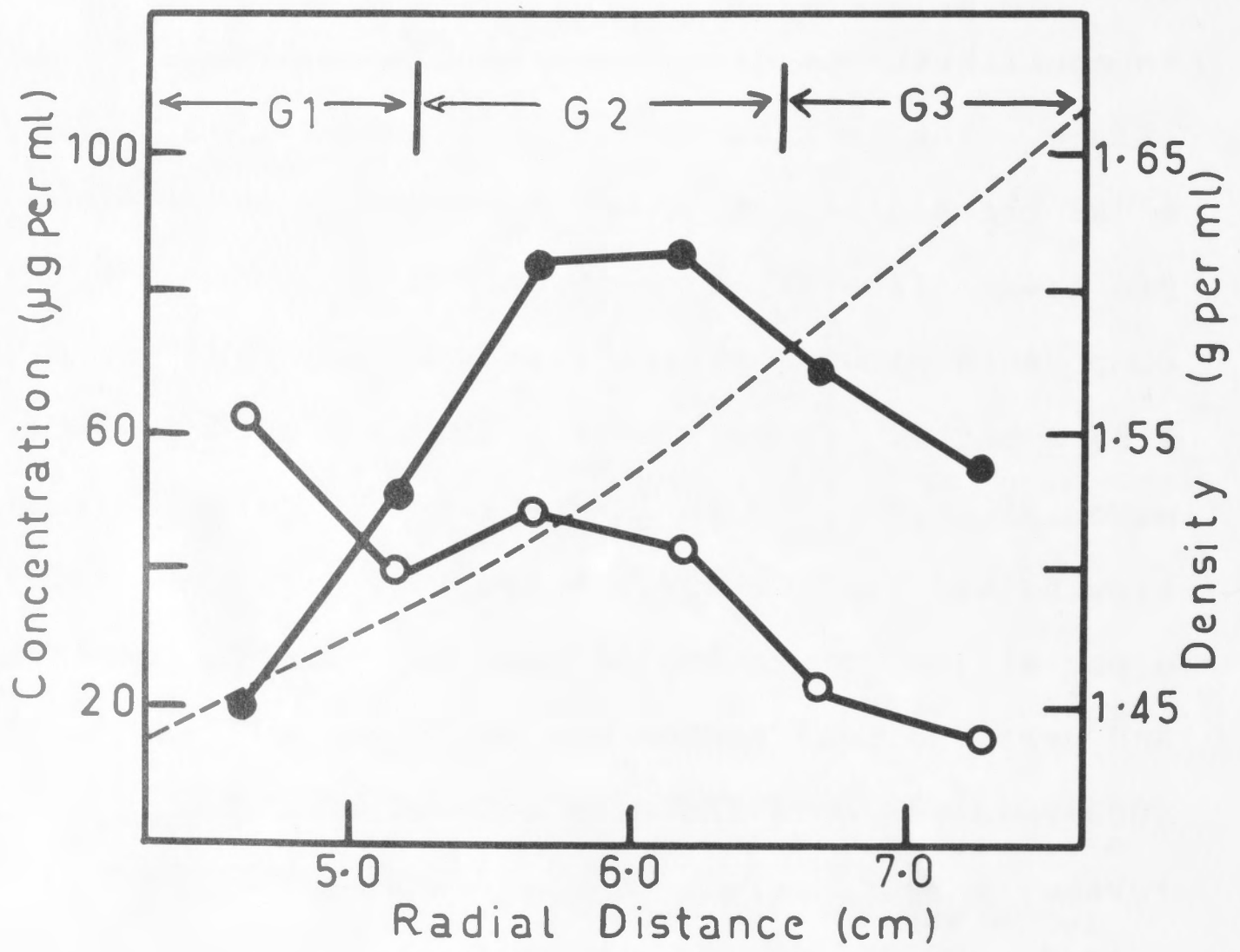


Fig.5:5. Distribution of uronic acid and sialic acid after centrifuging fraction P1 for 48 hours in a CsCl gradient in the no. 40 rotor. Details of the preparation are given in the text. The initial density was 1.5 g per ml. The density gradient is shown by the line - - - -, uronic acid by ●——●, sialic acid by O——O.

The material which accumulated near the meniscus (density 1.45 g per ml) contained significant amounts of sialic acid but little or no uronic acid. Material containing both uronic acid and sialic acid banded near the centre of the tube (density 1.49 - 1.57 g per ml). Consequential to these findings a preparative experiment was carried out.

Fractions obtained from several tubes were pooled as indicated in fig 5:5, dialysed free from CsCl, concentrated by ultrafiltration and stored at -20°. These fractions were designated G1, G2 and G3. Fraction G3 was not further investigated.

5:1:3. EXAMINATION OF FRACTION G1.

A detailed chemical analysis of fraction G1 is given in table 5:1, together with that of fractions G2 and P1.

Fraction G1 is composed largely of protein. The amino acid profile shows this to be somewhat more basic than the other fractions described having a slightly increased proportion of lysine. The carbohydrate moiety is composed of hexosamine, sialic acid and hexose. No uronic acid could be detected in 1.3 mg of material. By analogy with the material isolated by Radhakrishnam - urthy et al. (1964) this material was considered to be a glycoprotein or a mixture of glycoproteins. However

Table 5:1. Chemical Analysis of Fractions G1, G2 and P1.

ANALYSIS (mg per 100 mg dry weight)	P1	G1	G2
Uronic acid	11.79	-	19.64
Hexosamine	11.23	3.19	15.48
Glucosamine	7.88	2.81	12.63
Galactosamine	3.35	0.38	2.85
Sialic acid	5.15	3.85	5.99
Hexose[a]	11.26	6.69	6.56
Sulphate[b]	2.22	3.17	3.34
Protein[c]	56.95	92.62	56.17
Protein[d]	45.82	69.51	45.84
Molar Ratio, Hexosamine = 1.00			
Uronic acid	0.97	-	1.17
Hexosamine	1.00	1.00	1.00
Glucosamine	0.70	0.88	0.82
Galactosamine	0.30	0.12	0.18
Sialic acid	0.26	0.70	0.22
Hexose	0.25	2.08	0.42
Sulphate	0.37	1.85	0.40
Amino Acids (μmoles per 100 μmoles amino acid determined)			
Lys	7.8	9.6	7.0
His	1.9	2.2	2.1
Arg	4.7	4.9	4.8
Asp	10.4	10.1	10.7
Thr	6.6	5.5	7.1
Ser	6.8	6.5	7.1
Glu	15.5	13.9	15.7
Pro	5.6	5.4	5.5
Gly	7.2	6.6	7.7
Ala	8.5	8.8	7.8
CyS	1.3	2.2	1.2
Val	5.8	6.5	6.0
Met	1.1	1.3	1.1
Ile	3.3	4.1	3.3
Leu	7.2	8.0	6.4
Tyr	2.8	0.9	2.9
Phe	3.3	3.3	3.4

[a], Galactose; [b], as SO_4^{2-}; [c], determined by method of Lowry et al., (1951); [d], calculated from amino acid analysis.

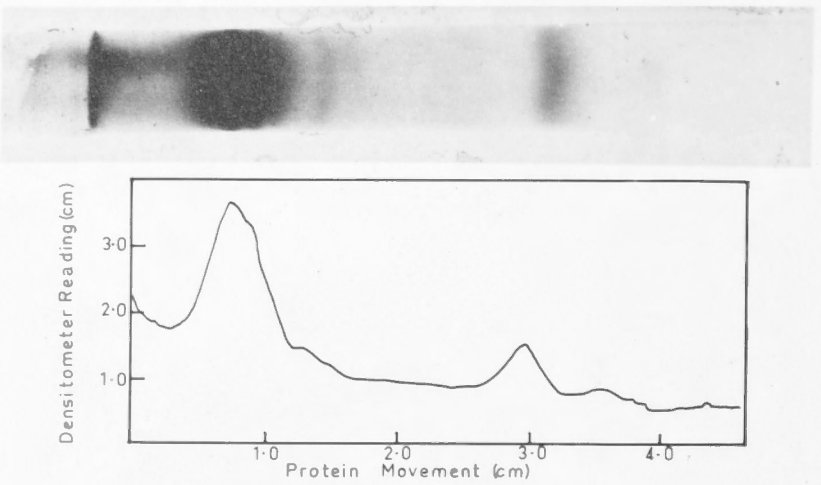


Fig. 5:6. Polyacrylamide gel electrophoresis of fraction G1. The stained column was photographed and the negative measured on a Beckman-Spinco Analytrol film densitometer.

a rather surprising feature of the carbohydrate moiety in fraction G1 is the high sulphate content, the molar ratio is higher than that of the total hexosamine present. Berenson and Fishkin (1962) found only a very small sulphate content (less than 0.2 percent) in their glycoprotein preparation from bovine aorta. The value found in this instance was 3.17 percent. No conclusions regarding this high sulphate content have yet been reached, however there is the possibility that a hitherto unrecog - nized carbohydrate component might be present. Further purification of this material is required before any conclusions can be made. Electorophoresis on polyacryl- amide shows that at least four components are present in fraction G1 (fig 5:6). However the greater proportion of the material is contained within a single band and it is not unreasonable to suppose that the chemical compos - ition of the mixture is representative of the major component. Similar findings were reported by Radhakrish - namurthy et al. (1964) when a crude preparation of bovine aorta glycoprotein was electrophoretically examined on polyacrylamide gel. It is also probable that this preparation is contaminated with some material of fraction G2 since it is unlikely that a complete separation of these two materials could have been obtained during a singe density gradient centrifugation; the resolution

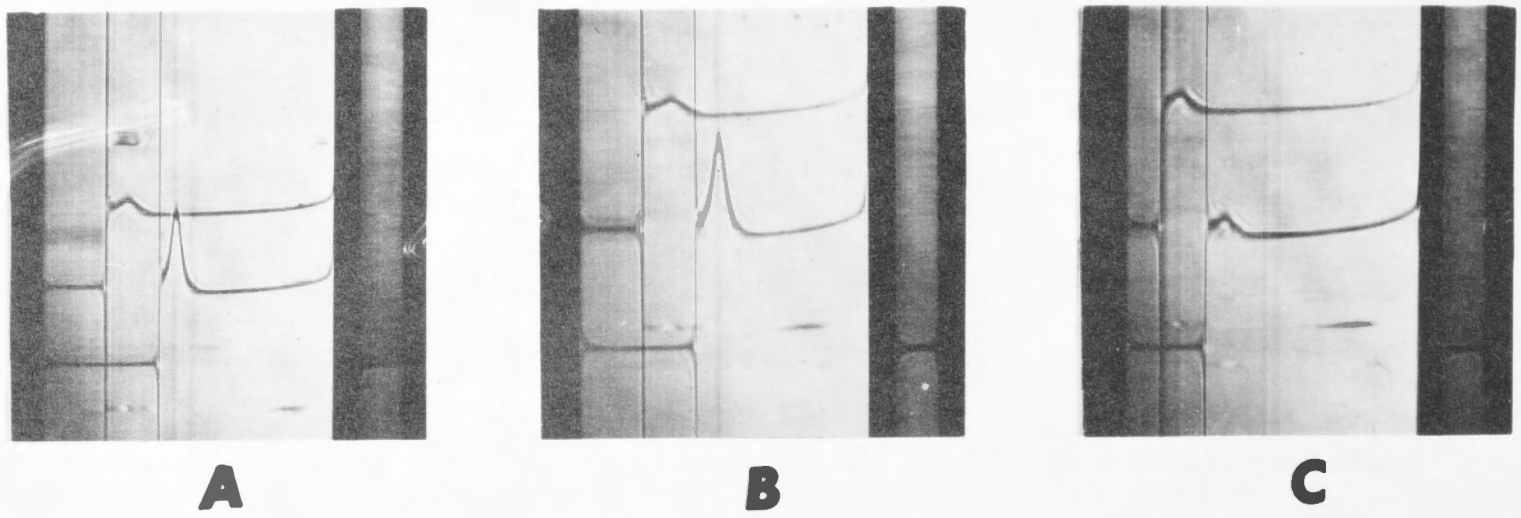


Fig. 5:7. Typical velocity sedimentation patterns of Fraction G2. Speed in all cases 50,740 rpm. (A). Bottom 0.119 g per dl. Top 0.053 g per dl. Phase plate angle 70°. 32 min. after reaching speed. (B) Bottom 0.119 g per dl. Top 0.053 g per dl. Phase plate angle 60°. 30 min. after reaching speed. (C) Bottom 0.024 g per dl. Top 0.021 g per dl. 18 min after reaching speed.

between these two fractions being small, see fig 5:5.

5:1:4. EXAMINATION OF FRACTION G2.

Chemical analysis of fraction G2 (table 5:1) points to this preparation being a mixture of low sulphated glycosaminoglycuronoglycan proteins. In chapter 4, section 4:3 it was suggested that hyaluronic acid and dermatan sulphate might be present in fraction P1. Thus the presence of a poorly sulphated glycosaminoglycurono - glycan protein could indicate that a hyaluronic acid protein has been isolated in the fraction G2. The presence of sialic acid and hexose could result from contamination from fraction G1, however sialic acid and hexose are known to be contained in cartilage glycosaminoglycuronoglycan protein preparations (Anderson, 1961, 1962).

The protein content of fraction G2 is high when compared with similar glycosaminoglycuronoglycan proteins isolated from other tissues, for example, the protein content of bovine nasal cartilage proteinpolysaccharide is less than 15 percent (Luscombe and Phelps, 1967a). However it is impossible to determine, from these chemical analyses, whether this protein is all associated with glycosaminoglycuronoglycan material or if a large part of it is due to comtamination (possibly contamin - ation from fraction G1). The amino acid profile is

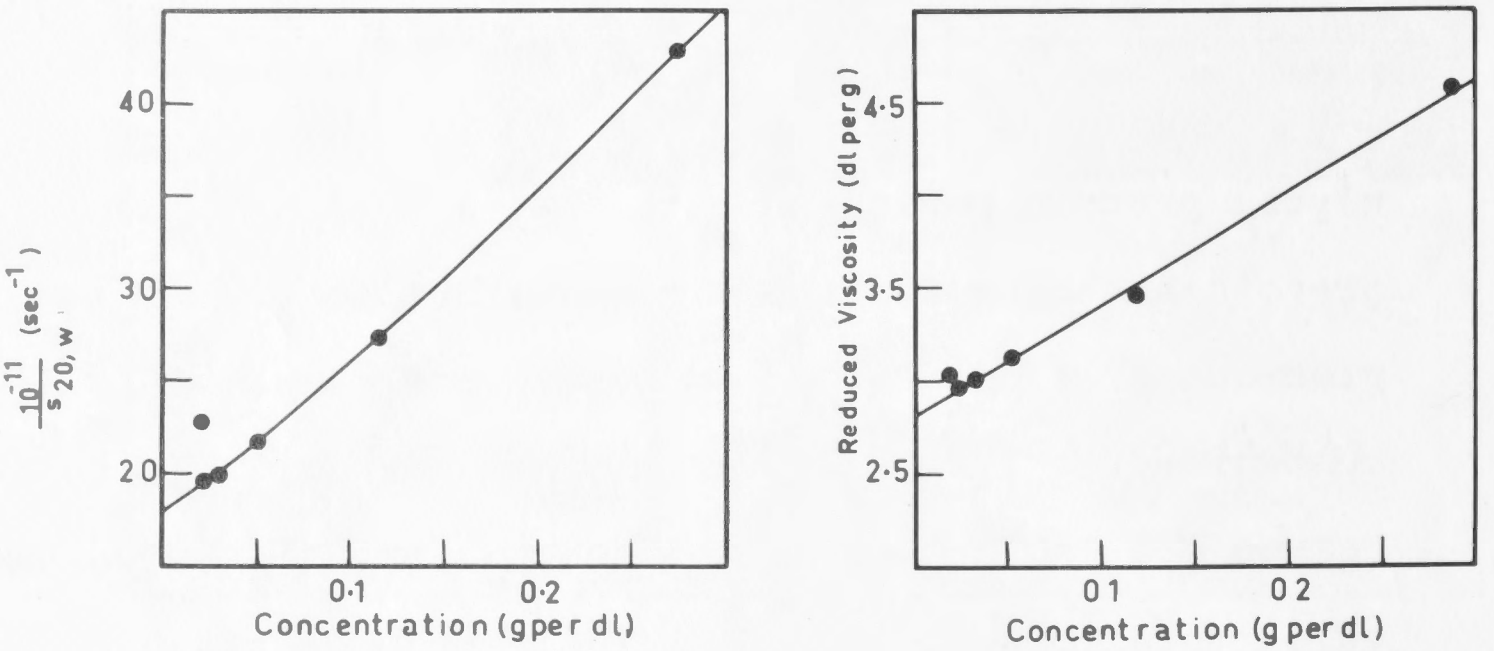


Fig. 5:8. The effect of the concentration of fraction G2 on (a) the sedimentation coefficient and (b) the viscosity.

similar to that of fraction P1 but is slightly less basic that that of G1. However it is not unlike that found in the hyaluronic acid protein preparation descr - ibed by Preston et al. (1965).

Examination of fraction G2 in the ultracentrifuge showed this preparation to move as a single boundary over the concentration range 0.02 to 0.29 g per dl. Typical velocity sedimentation patterns of fraction G2 are illustrated in fig 5:7. The dependence of the sedimentation coefficient upon concentration is shown in fig 5:8. Also included in this figure is the dependence of reduced viscosity upon concentration. Values of the reduced viscosity and the sedimentation coefficient at zero concentration are respectively 2.83 dl per g and 6.25×10^{-13} sec.

Infrared absorption spectroscopic studies of fraction G2 showed this material to be poorly sulphated (Orr,1954). Such findings are in agreement with the chemical analysis.

Chemical analysis indicates that this material could contain hyaluronic acid, heparin or heparan sulphate since glucosamine is the predominant hexosamine present. The galactosamine then, could be associated with dermatan sulphate. To clarify this possibility an aliquot was precipitated on to cellulose using

cetylpyridinium chloride (batch method, Buddecke, et al., 1963). After removing the material that did not precipiate onto the cellulose, material was eluted from the cellulose first with 0.3M NaCl and then with increasing concentrations of $MgCl_2$. The results of these analyses are shown in table 5:2.

Table 5:2. Analysis of fraction G2 by precipitation onto cellulose using cetylpyridinium chloride (batch method, Buddecke et al., 1963).

ELUTING SOLVENT	URONIC ACID (Expressed as percentage of the total amount recovered)
1 % cetylpyridinium chloride	30.6
0.3M NaCl	39.8
0.3M $MgCl_2$	20.4
0.5M $MgCl_2$	9.2
0.75M $MgCl_2$	0.0
1.5M $MgCl_2$	0.0

Approximately one third of the material was not precipitated onto the cellulose. However a significant proportion of the material that was absorbed could be eluted with 0.3M NaCl, suggesting that this material is hyaluronic acid (Antonopoulos et al., 1961; Thunell et al., 1967). No material was eluted with 1.5M $MgCl_2$ suggesting the absence of heparin (Antonopoulos et al., 1964)

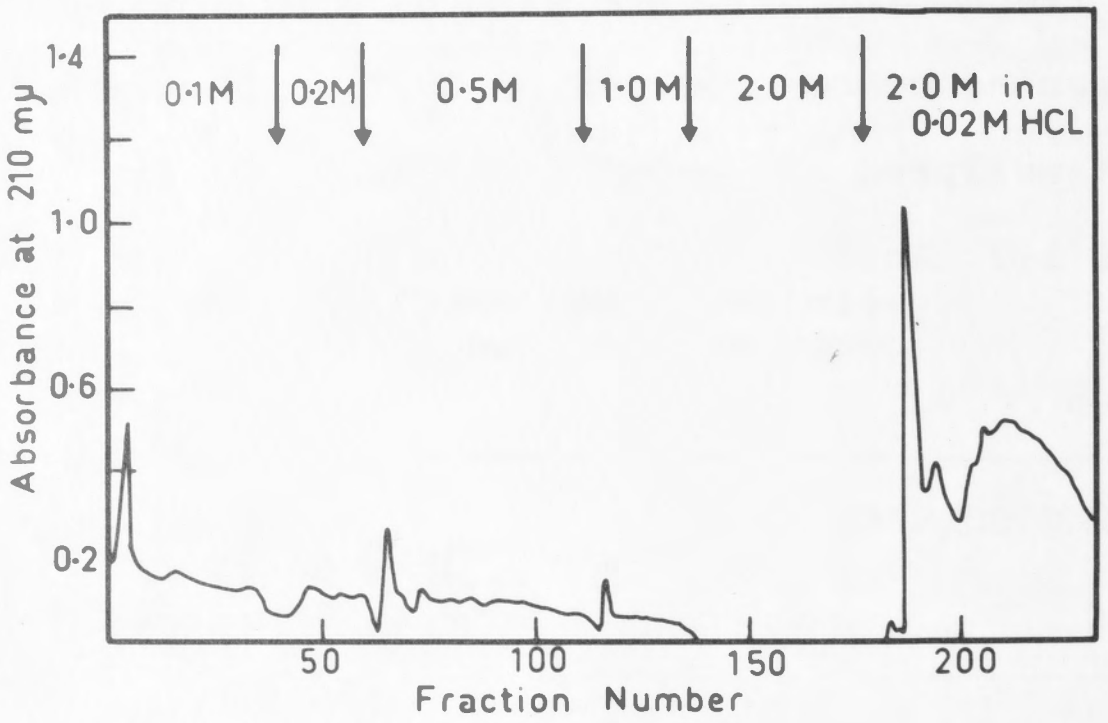


Fig. 5:9. Chromatography on DEAE-cellulose of fraction G2. Details of the preparation are given in the text. 3.1 mg in 5.0 ml of solution was applied to column (18.0 x 1.04 cm). Gradient elution was carried out discontinuously, the changes in composition of the eluting solvent are indicated by arrows. The elution pattern was determined by measuring the extinction of each fraction at 210 mμ.

The material eluted with 0.3M $MgCl_2$ might be heparan sulphate (Thunell et al., 1967) but it could also represent some overlap from the previous fraction. Dermatan sulphate if present could account for the material that has been eluted with 0.5M $MgCl_2$.

Chromatography of fraction P1 on DEAE-cellulose (section 5:1:1) resulted in three major fractions being isolated. To correlate fraction G2 with these findings an aliquot of this preparation (3:1 mg in 5.0 ml) was applied to a DEAE-cellulose column (18.0 x 1.04 cm). Material was eluted from the column by a discontinuous gradient of NaCl and finally with acidified NaCl. The flow rate was 23.5 ml per sq cm per hour, 10 ml fractions were collected. The elution pattern determined as previously described is illustrated in fig 5:9.

Approximately two thirds of the material was eluted with acidified NaCl, indicating that in the previous experiments (section 5:1:1) much of the glycosaminoglycuronoglycan protein material was eluted only with acidified NaCl. Material that was eluted with lower concentrations of salt can possibly be identified with fraction G1.

5:1:5. SUMMARY.

Equilibrium sedimentation of fraction P1 in a CsCl density gradient of initial density near 1.50 g per ml has resulted in a further separation of this material into a fraction G1 with a density near 1.45 g per ml and a fraction G2 with density near 1.54 g per ml.

Fraction G1 appears to be a mixture of glycoproteins, resembling the glycoprotein material isolated by Radhak - rishnamurthy et al. (1964). However the chemical composition of the carbohydrate portion differs somewhat from the preparation described by these workers in that it contains an unusually high sulphate content. Electrophoresis on polyacrylamide gel shows this material to be heterogeneous and while approximately two-thirds of the material was contained in a single band no definite conclusions can be drawn with regard to the carbohydrate composition, although it is possible that this material could represent a new proteinpolysaccharide.

In order to obtain a clearer conception of this material it is necessary that further purification of this material be made. This could possibly be achieved by a further density gradient centrifugation at a lower initial density, say 1.43 to 1.45 g per ml. This would result in the elimination of any non-specific protein that may still be contained in the preparation and it

might result in this this proteinpolysaccharide material being banded. Purification might also be attempted using polyacrylamide gel electrophoresis AND/or chromatography on DEAE-cellulose. The latter method perhaps could separate the highly sulphated components present since they are more likely to be retained by the column.

From chemical analysis and from precipitation onto cellulose with cetylpyridinium chloride it would appear that fraction G2 contains a hyaluronic acid protein. However, these analyses also suggested the presence of heparan sulphate and dermatan sulphate. To further clarify these findings, a detailed analysis of each fraction eluted from the cellulose after precipitation with cetylpyridinium chloride is essential. It is feasible that further purification of this fraction is possible after recentrifugation in a CsCl density gradient. It is possible that the material would require a "double recentrifugation", firstly at a lower initial density, say 1.45 g per ml; this centrifugation being aimed at removing any contaminating non-specific protein material that may or may not be associated with fraction G1 and secondly a recentrifugation at a lower concentration of proteinpolysaccharide material; this might result in a finer resolution of the various glycosaminoglycuronoglycan proteins that appear to be

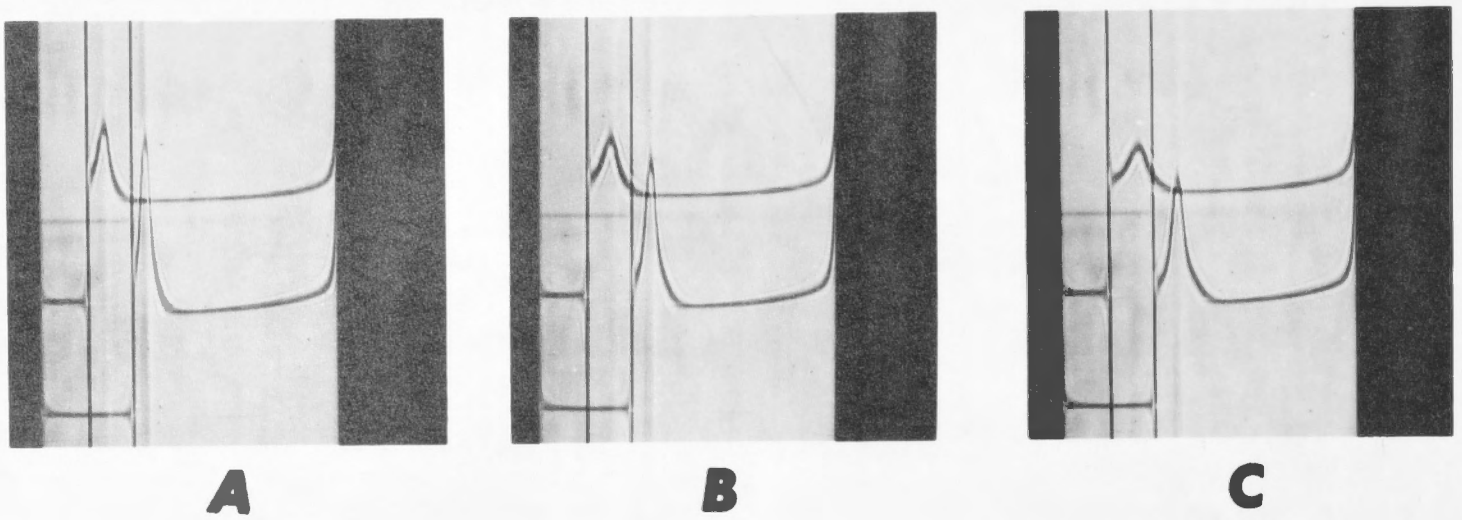


Fig. 5:10. Typical velocity sedimentation patterns of Fraction P3. Bottom 0.35 g per dl. Top 0.17 g per dl. Phase plate angle 60°. (A) 12 min. (B) 20 min and (C) 28 min. after reaching speed of 50,740 rpm.

present.

It would be of little advantage to endeavour to further purify fraction G2 on DEAE-cellulose since this fraction can only be eluted from the column with acidified NaCl. Such acid conditions must be avoided if the aim of the experiment is to study the macromolecular nature of the particular proteinpolysaccharide.

5:2. EXAMINATION OF FRACTION P3.

Fraction P3 was obtained from the high density region (1.65 - 1.75 g per ml) after a crude extract from porcine aorta was centrifuged to equilibrium in a CsCl density gradient of initial density 1.63 g per ml (section 4:3).

Chemical analysis of this preparation (4:3) showed it to contain uronic acid (17.5 percent), galactosamine (11.3 percent), glucosamine (2.4 percent), hexose (18.8 percent) and protein, approximately 10 percent. From these findings and from the results obtained after precipitation of an aliquot of fraction P3 onto cellulose with cetylpyridinium chloride it was suggested that this material contained two or more glycosaminoglycurono - glycan protein components (section 4:3).

Examination of this material during velocity sedim - entation in the ultracentrifuge showed that it moved as a single boundary over the concentration range 0.17 to

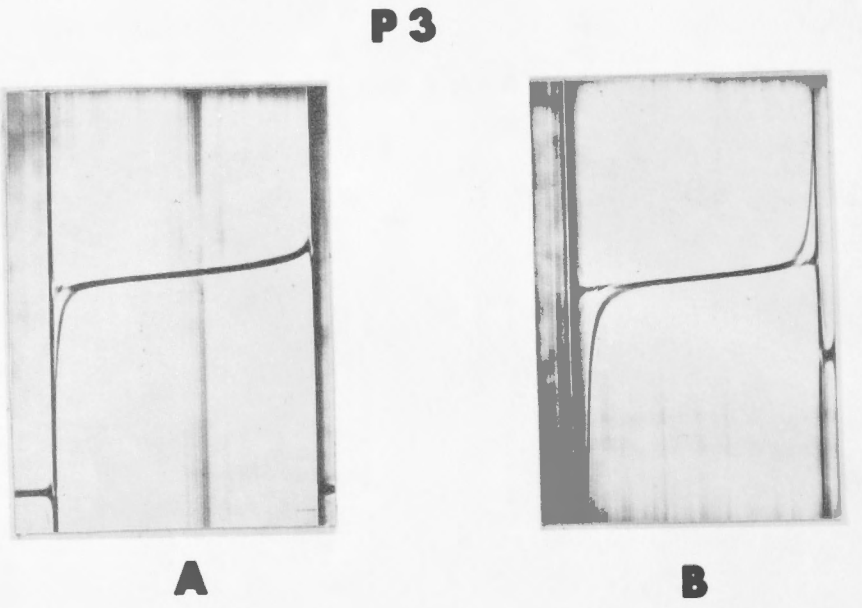


Fig.5:11. Density gradient sedimentation equilibrium diagrams of Fraction P3. Details of preparation are given in the text. The initial density was 1.796 g per ml. The phase plate angle was 60o. (A) 18.6 hours after reaching speed of 44,770 rpm. (B) 12.3 hours after reducing speed to 29,500 rpm.

0.35 g per dl. This, once more, emphasizing the care that must be exercised in the interpretation of sedim - entation velocity boundaries (see fig. 5:10).

5:2:1. EXAMINATION OF FRACTION P3 IN A DENSITY GRADIENT.

Having had a degree of success in further fraction - ating fraction P1 by recentrifuging in a density gradient of initial density close to that at which the material had reached equilibrium it was decided to investigate fraction P3 in a CsCl gradient that had an initial density of 1.8 g per ml, since this would provide a density distribution near to that in which this fraction had reached equilibrium.

5:2:1:1. Analytical Density Gradient Centrifugation of Fraction P3.

A solution of fraction P3 adjusted to a density of 1.796 g per ml with CsCl was centrifuged to equilibrium at 44,770 rpm in the model E Beckman-Spinco ultracentri- fuge. The resulting density ranged from 1.732 g per ml at the meniscus to 1.876 g per ml at the cell bottom. The distribution of material within this gradient is shown in fig 5:11 A. Material accumulated only near the meniscus. However, when the rotor was slowed down to 29,500 rpm the resulting density distribution was 1.768 g per ml at the meniscus to 1.827 g per ml near the cell bottom and material was seen to have accumulated

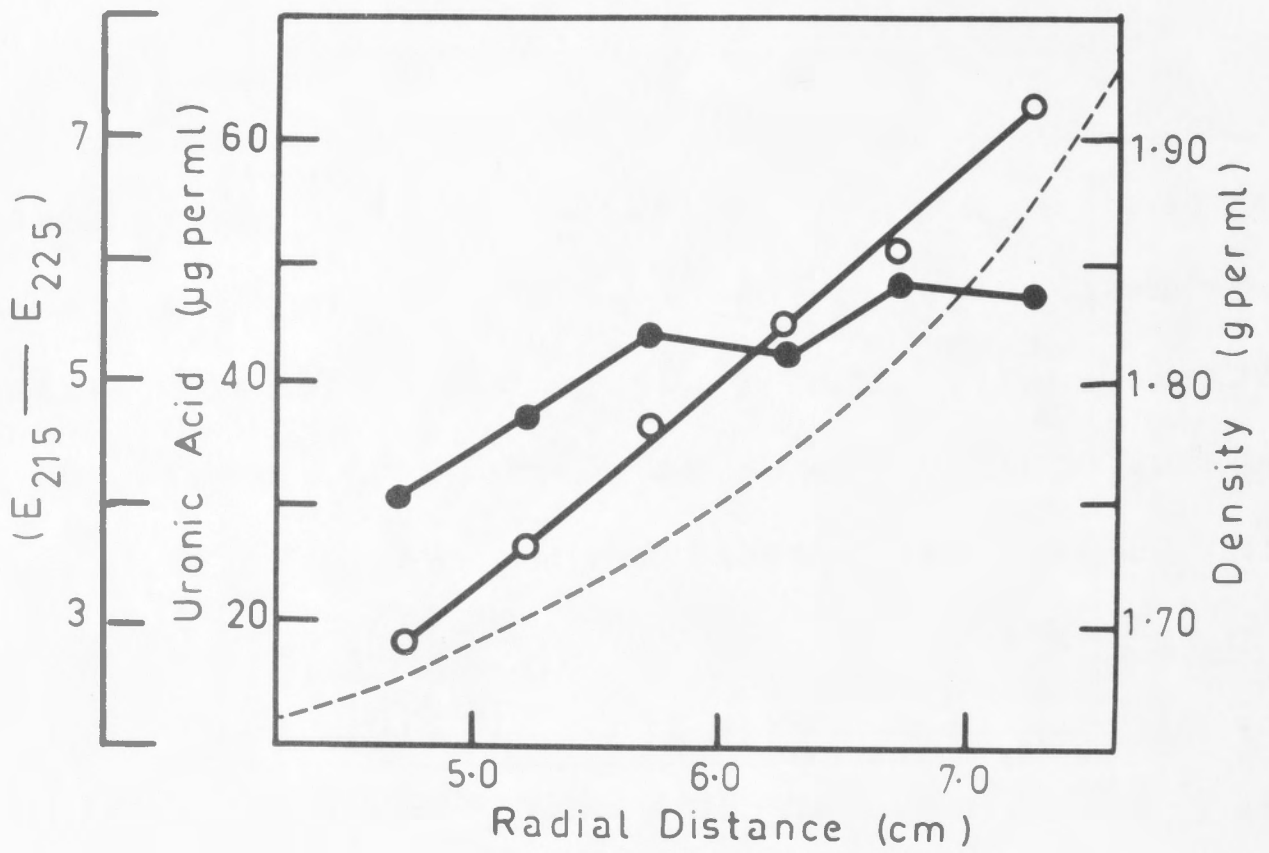


Fig. 5:12. Distribution of uronic acid and protein after centrifuging Fraction P3 for 48 hours in a CsCl density gradient in the no 40 rotor. Details of the preparation are given in the text. The initial density was 1.79 g per ml. The density gradient is shown by the line - - - -, uronic acid o——o, protein ●——●.

also at the bottom of the cell (fig 5:11 B), indicating the presence of material with density near 1.83 g per ml but the absence of material with density higher than 1.86 g per ml. Such findings are indicative of density heterogeneity; to clarify these results a preparative experiment was carried out.

5:2:1:2. Preparative Gradient Centrifugation of Fraction P3.

An aliquot of a solution of Fraction P3 adjusted to an initial density of 1.79 g per ml was centrifuged for 48 hours at 35,000 rpm in the preparative ultracentrifuge (no. 40 rotor). After this time a sample tube was removed and the position of material within the gradient determined by chemical means. The results of these analyses are illustrated in fig 5:12. The distribution of uronic acid and of protein was continuous throughout the gradient suggesting the presence of a continuum of molecules with only slightly different densities. This prohibits the further useful separation of fraction P3 by density gradient centrifugation.

5:2:2. FRACTIONATION USING ALIPHATIC AMMONIUM IONS.

Previously when fraction P3 was precipitated onto cellulose using cetylpyridinium chloride some degree of separation was obtained (section 4:3); of the material eluted with $MgCl_2$ most of it was recovered with 0.5M and 0.75M $MgCl_2$. This fractionation was therefore repeated using larger amounts of material.

An aliquot of fraction P3 was precipitated onto cellulose with cetylpyridinium chloride using the batch process (Buddecke et al., 1963). Material that was not precipitated onto the cellulose was removed by washing with 1 percent cetylpyridinium chloride. The complexes were then eluted from the cellulose with increasing concentrations of $MgCl_2$ after an initial elution with 0.3M NaCl. The proteinpolysaccharides were recovered from their cetylpyridinium chloride complexes by precipitation with ethanol and potassium acetate. The precipitates were then redissolved in water and again precipitated with ethanol and potassium acetate in order to remove the last traces of $MgCl_2$. (Antonopoulos et al., 1961). Each isolated precipitate was then dissolved in a known volume of water and chemical analyses were performed; the results of these analyses are shown in table 5:3. An analysis of fraction P3 is shown for comparison.

The material that was not precipitated onto the cellulose is similar in composition to fraction P3 with the exception that the amount of glucosamine has been significantly reduced. On the other hand the material eluted with 0.3M NaCl contained mainly glucosamine and protein with small amounts of uronic acid and hexose. While material eluted with 0.3M $MgCl_2$ contained in

Table 5:3. Analysis of Fraction P3 by Precipitation onto Cellulose with Cetylpyridinium Chloride (batch process, Buddecke et al.,1963).

ANALYSIS	1 % CPC[a]	µg per fraction				
		0.3M NaCl	0.3M $MgCl_2$	0.5M $MgCl_2$	0.75M $MgCl_2$	P3
Uronic acid	2640	43	333	848	2000	
Hexosamine	2175	496	202	624	1950	
Glucosamine	30	496	170	328	125	
Galactosamine	2145	-	31	296	1825	
Hexose[b]	2991	43	1951	564	172	
Protein[c]	1875	264	185	440	1237	
	Molar ratio - Hexosamine - 1.00					
Uronic acid	1.12	0.08	1.52	1.26	0.94	1.18
Hexosamine	1.00	1.00	1.00	1.00	1.00	1.00
Glucosamine	0.02	1.00	0.88	0.51	0.06	0.17
Galactosamine	0.99	-	0.20	0.46	0.94	0.83
Hexose	1.37	0.87	9.55	0.88	0.09	1.37

[a], Cetylpyridinium chloride; [b], estimated as galactose;
[c], determined by method of Lowry et al. (1951).

addition to glucosamine and protein approximately ten times the amount of hexose. These two fractions then have surprising chemical compositions. No attempt was made at this stage to identify the hexoses present, but since it was shown in chapter 4, section 4, that the main hexoses present in fraction P3 were glucose, fucose and galactose it is reasonable to assume that these hexoses are present in the material eluted with 0.3M $MgCl_2$. It is possible that keratan sulphate is present in this fraction, however from the molar ratio of glucosamine to hexose, it is seen that there is still a large proportion of hexose not associated with any hexosamine. The material eluted with 0.75 M $MgCl_2$ contains equimolar amounts of uronic acid and galactosamine and near equimolar amounts of glucosamine and hexose. This material is similar in composition to bovine nasal cartilage proteinpolysaccharide, i.e. it is a chrondroitin sulphate/keratan sulphate protein material; it is also similar to the material isolated by Buddecke et al. (1963) from human aorta. Material eluted with 0.5M $MgCl_2$ contains equimolar amounts of glucosamine and galactosamine in addition to significant amounts of uronic acid and hexose; it is probable that this fraction is contaminated with material eluted with 0.3 M and 0.75 M $MgCl_2$.

Only that material eluted with 0.75 M $MgCl_2$ was further investigated at this stage, since it was this material that was required to compare with the bovine nasal cartilage proteinpolysaccharide; for brevity it was designated P3 0.75.

5:2:3. EXAMINATION OF FRACTION P3. 0.75.

A detailed analysis of this fraction is given in table 5:4 together with that of fraction P3. The physical chemical properties of this preparation will be discussed in chapter 7.

The carbohydrate moiety of fraction P3 0.75 contains mainly uronic acid and galactosamine, these being present in approximately equimolar proportions. Similarly glucosamine and hexose are present in approximately equimolar amounts but in smaller proportions. The molar ratio of sulphate would indicate that most of the hexos - amine is sulphated. The protein moiety is not signifi - cantly different from that of fraction P3, it represents approximately 12 percent of the dry weight and is similar to that obtained from bovine nasal cartilage and human aorta (Buddecke et al., 1963). Except for a decrease in the amount of glycine the amino acid profile does not differ much from fraction P3 and is similar to that of a chondroitin sulphate protein preparation from nasal cartilage described by Pal et al. (1966).

Table 5:4. Chemical Analysis of Fractions P3 and P3.0.75.

ANALYSIS (mg per 100 mg dry weight)	P3	P3.0.75
Uronic acid	17.50	19.85
Hexosamine	13.69	19.49
Glucosamine	2.39	1.24
Galactosamine	11.30	18.25
Hexose[a]	18.84	1.72
Sulphate[b]	7.20	9.26
Protein[c]	12.56	12.37
Protein[d]	8.40	
Mole ratio. Hexosamine - 1.00		
Uronic acid	1.18	0.94
Hexosamine	1.00	1.00
Glucosamine	0.18	0.06
Galactosamine	0.82	0.94
Hexose	1.37	0.09
Sulphate	0.98	0.89
Amino acids (μmoles per 100 μmoles of amino acid determined).		
Lys	3.6	4.1
His	1.7	1.9
Arg	2.3	2.4
Asp	8.7	8.0
Thr	8.8	10.8
Ser	9.7	10.8
Glu	14.8	14.5
Pro	7.0	5.8
Gly	17.5	8.2
Ala	7.8	8.9
CyS	trace	trace
Val	5.9	6.7
Met	trace	1.8
Ile	3.3	4.2
Leu	6.2	7.4
Tyr	0.5	1.1
Phe	2.2	3.4

[a], as galactose; [b], as SO_4^{2-}; [c], determined by the method of Lowry et al.(1951); [d], calculated from the amino analysis.

A summary of the main bands observed in the infrared absorption spectra is given in table 5:5. Both chondroitin 4-sulphate and chondroitin 6-sulphate would appear to be present although chondroitin 6-sulphate would appear to be the more prominant species. Similar findings were reported by Buddecke et al. (1963).

Table 5:5. The Infrared Spectra of Fractions P3 and P3 0.75

Chondroitin 4-SO_4 cm^{-1}	Chondroitin 6-SO_4 cm^{-1}	P3 cm^{-1}	P3.0.75 cm^{-1}
-	1000	1055, 960	1055, 960
928	-	-	-
855	-	-	-
-	825	820	825
-	775	775	775
725	-	725	725

5:2:4. SUMMARY.

Investigation of fraction P3 by further centri - fugation in a density gradient revealed that this material was considerably density heterogeneous. A separation of the components of this material was obtained by precipitation on cellulose with aliphatic ammonium ions and elution with increasing concentrations of salt.

The results of this analysis showed that fraction P3 contained some rather unusual components. The main feature of these components being a very high hexose content. The fraction eluted with 0.75M $MgCl_2$ was a chondroitin sulphate protein material. Chondroitin 4-sulphate and chondroitin 6-sulphate were both present as also was hexose and glucosamine. These latter two components suggest the presence of keratan sulphate. Such findings agree with those of Buddecke (1960) but are in disagreement with those of Antonopoulos et al. (1965); these workers were unable to identify keratan sulphate in aortic tissue digests that had been fractionated with quaternary ammonium ions. However this preparation has a chemical composition similar to that of the proteinpolysaccharide of bovine nasal cartilage and although, as will be shown in the next chapter, it has a molecular weight considerably smaller than cartilage proteinpolysaccharide, the molecular weight found in this instance is significantly higher than that reported by Buddecke et al. (1963).

In general the fractionation of fraction P3 using quaternary ammonium ions has revealed an extremely complex pattern of proteinpolysaccharide components to be present. Complete clarification of each component using the above mentioned technique is doubtful. However the partial

fractionation that has been obtained could result in other methods of separation being applicable. Further since the chemical analyses presented in this work were not exhaustive, it might well be that a more detailed chemical investigation would disclose a suitable approach upon which further methods of separation might be followed.

The initial aim of these investigations was to isolate all the proteinpolysaccharide species present in aortic tissue. The experimental results presented in this chapter have revealed that this material is very much more complex than was originally envisaged. The situation is further complicated by the difficulty in obtaining sufficient material upon which investigations can be made. The supply of aortic specimens from the abattoirs was limited to between 50 to 80 per week, thus in order to obtain sufficient crude proteinpolysaccharide extract, several preparations were required. Further to obtain sufficient quantities of the fractions isolated during density gradient centrifugation, several equilibrium centrifugations were necessary. For these reasons it was decided to limit the work at this stage to the identification and characterization of only one type of proteinpolysaccharide species, namely that represented by fraction P3. 0.75.

In order to make a comparison of this fraction with a similar proteinpolysaccharide obtained from another source, a detailed investigation of the proteinpoly - saccharide from nasal cartilage was undertaken. The results of this investigation are presented in the following chapter, and some physico-chemical properties of the preparations are compared in chapter 7.

Chapter 6.

The Proteinpolysaccharide of Bovine Nasal Cartilage.

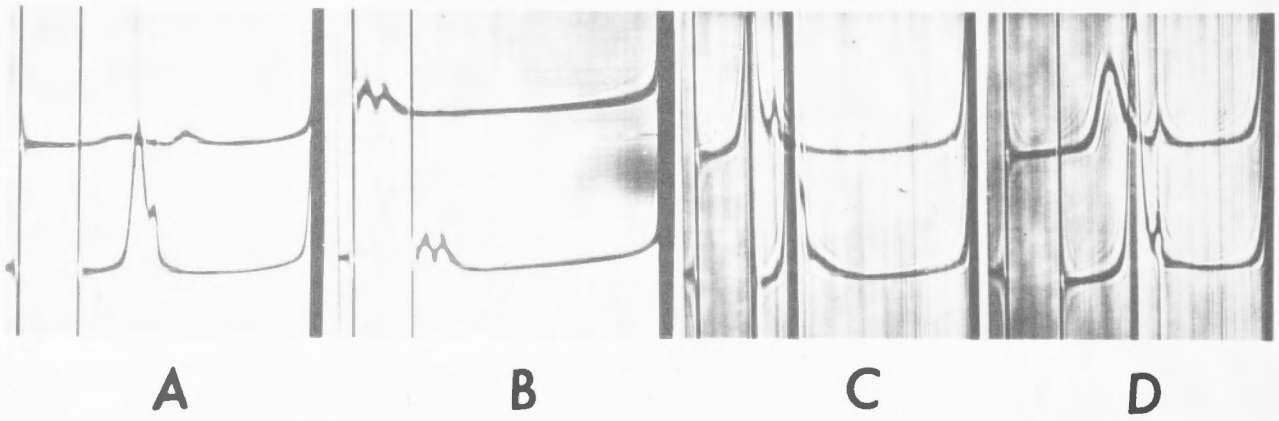


Fig. 6:1. Sedimentation velocity patterns of fraction I. Details of the preparation are given in the text. The speed in all cases was 50,740 rpm, and the schlieren phase plate angle was 60°, except in B where it was 55°. Experiments were carried out in standard.* Direction of sedimentation is from left to right. A, top 0.149 g per dl; bottom 0.290 g per dl; 29 min after reaching speed. B, top 0.057 g per dl; bottom 0.070 g per dl; 2 min after reaching speed. C, top 0.335 g per dl; bottom 0.450 g per dl; 34 min after reaching speed. Note the hypersharp boundaries. D, the same solution as in C but 62 min after reaching speed and showing resolution of the hypersharp boundaries.

*errata, standard buffer

6:1. HETEROGENEITY OF CARTILAGE PROTEINPOLYSACCHARIDE.

Mention has already been made, chapter 1 section 1:3:3:3. of the heterogeneity of nasal cartilage proteinpolysaccharide preparations. This heterogeneity is manifested both by variation in particle weight and shape and by variations in particle density. Because of the nature of these variations it would seem that further characterization of the components of cartilage proteinpolysaccharide preparations, by methods which depend upon these types of variations should be attempted.

Except when otherwise stated the nasal cartilage proteinpolysaccharide solutions were prepared as described previously (section 2:1:4:2). Such preparations have been designated, fraction 1.

6:1:1. VELOCITY SEDIMENTATION.

Typical schlieren patterns of the sedimentation of crude extracts of proteinpolysaccharide are shown in fig. 6:1A, B, C and D. The presence of two sedimenting boundaries can be clearly seen. At very low concentrat - ions of proteinpolysaccharide (0.06 g per dl, fig 6.1B) the two boundaries were clearly resolved after only two minutes at 50,740 rpm. At higher concentrations (0.33 to 0.45 g per dl) the resolution was not so obvious because of hypersharpness (fig 6:1C), but after one hour at 50,740 rpm the fast and slow moving boundaries could

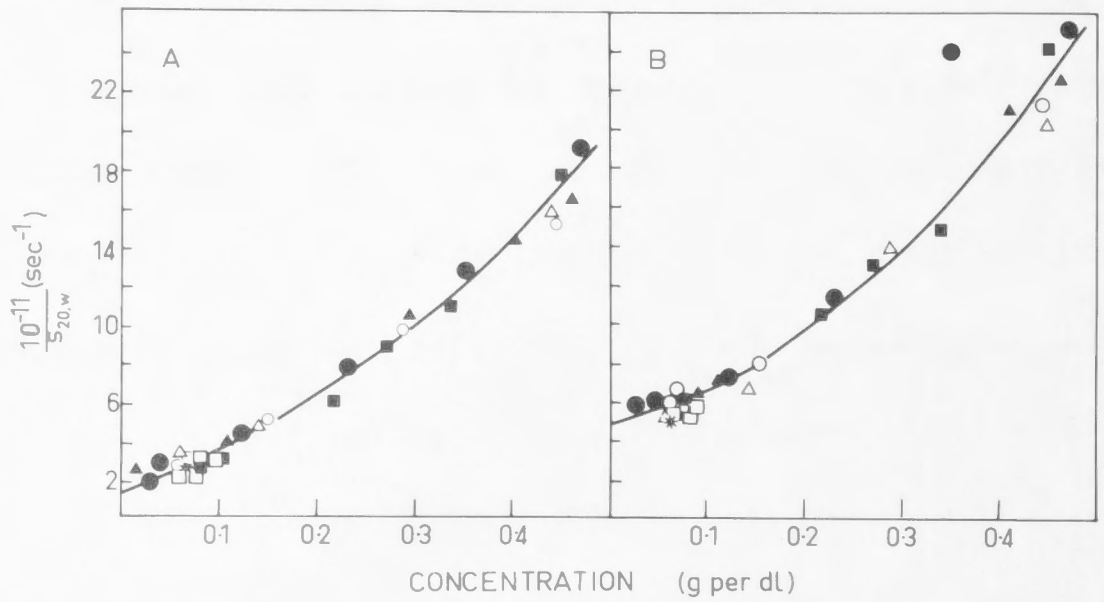


Fig. 6:2. The effect of total concentration of the protein polysaccharide on the sedimentation coefficients of the fast and slow sedimenting components. A, the faster sedimenting components; and B, the slower sedimenting components of various proteinpolysaccharide solutions from bovine nasal cartilage. The solutions were prepared as follows:

Preparation 1 from nasal cartilage as described in the Experimental (section, 2:1:4:2:1 fraction I) the minced cartilage had been stored for varying periods (up to 12 months) at -20°, 0; Preparation 2, as in Preparation 1 but prepared solutions were stored at -20° for 6 weeks, △ and 8 weeks □, before examination; Preparation 3 as for preparation 1 but without precipitation of the proteinpolysaccharides with ethanol and potassium acetate, *; Preparation 4 freshly collected nasal cartilage, examined immediately, ■; Preparation 5, from acetone dried cartilage exactly as described by Gerber et al. (1960), ▲; Preparation 6, from nasal cartilage proteinpolysaccharide by sedimentation to equilibrium in a CsCl density gradient greater than 1.65 was examined ●.

be easily observed (fig 6:1D).

The effect of concentration on the sedimentation coefficients of these components is shown in fig 6:2A, B. The values recorded in this figure have been obtained from:

Preparation 1, proteinpolysaccharide solutions which were prepared from nasal cartilage as described in the experimental section, (2:1:4:2, fraction 1), the minced cartilage had been stored for varying periods (up to 12 months)at -20°;

Preparation 2, proteinpolysaccharide solutions prepared as for Preparation 1 but subsequently stored for (a) six weeks and (b) eight weeks at -20° before examination;

Preparation 3, a preparation prepared as for Preparation 1 but with the omission of the precipit - ation of the proteinpolysaccharide with ethanol and potassium acetate;

Preparation 4, a preparation of proteinpolysaccharide prepared from freshly collected nasal cartilage and examined immediately;

Preparation 5, preparation from acetone-dried cartilage carried out exactly as described by Gerber et al. (1960)

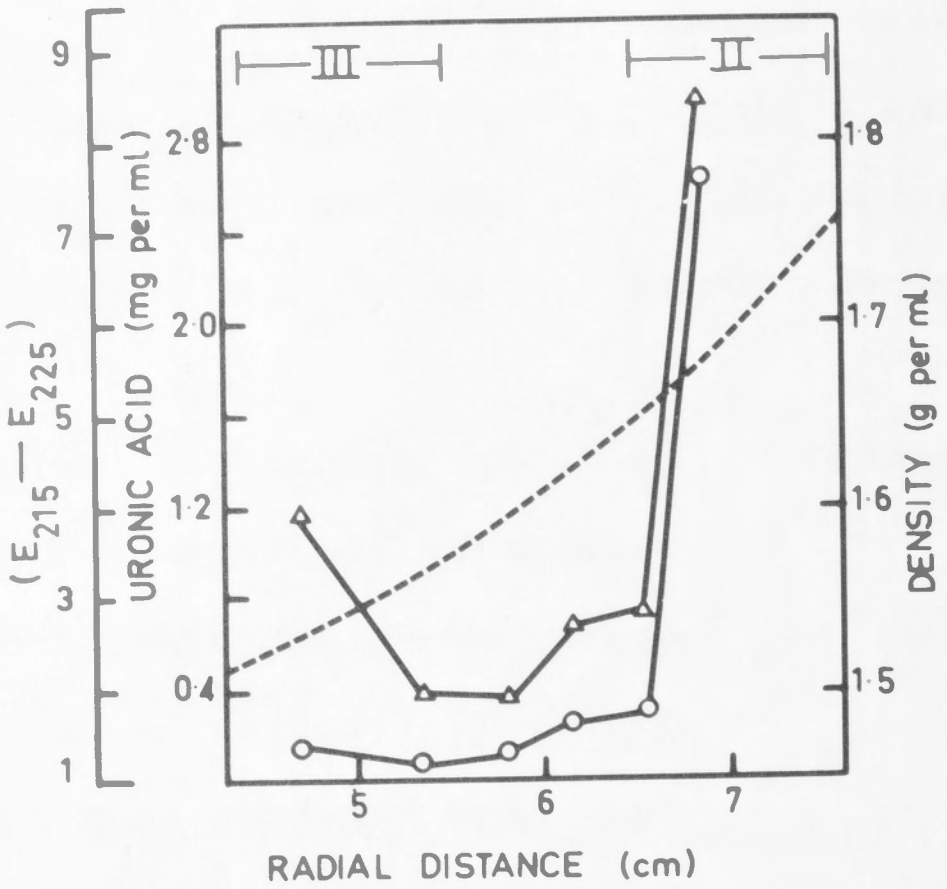


Fig.6:3. Distribution of uronic acid and protein after the proteinpolysaccharide, fraction I was centrifuged to equilibrium in a CsCl density gradient. The density gradient is shown by the line ——; uronic acid by O——O; and protein △——△. Sedimentation was continued for 48 hours at 35,000 rpm in the No. 40 rotor of a Beckman model L ultracentrifuge. Fractions II and III were obtained as illustrated.

The results illustrated in fig 6:2A, B indicate that the treatment prior to and subsequent to extraction have little effect upon the sedimentation properties of the molecules.

6:1:2. DENSITY HETEROGENEITY.

The proteinpolysaccharide material of nasal cartil - age has a partial specific volume near 0.55 g per ml (Mathews and Lozaityte, 1958) and thus should have a density near 1.8 g per ml. If glycoprotein or globular protein components are present in the preparation they will have a density less than 1.5 g per ml as indicated in chapter 5, section 5:1:3. Thus in order to visualize the behaviour of nasal cartilage proteinpolysaccharide in a density gradient, a solution of the proteinpolysaccha- ride (fraction 1) was adjusted to an initial density of 1.63 g per ml with solid CsCl and centrifuged to equilibrium at 35,000 rpm in the preparative ultra - centrifuge (rotor no. 40). In designing this experiment it was hoped that density heterogeneity, if present, could be correlated with the two boundaries observed in the velocity sedimentation experiments and/or such heterogeneity might result in the identification of a glycoprotein component.

The results of the experiment are shown in fig 6:3. The distribution of material within the gradient is indicated by the distribution of uronic acid and protein.

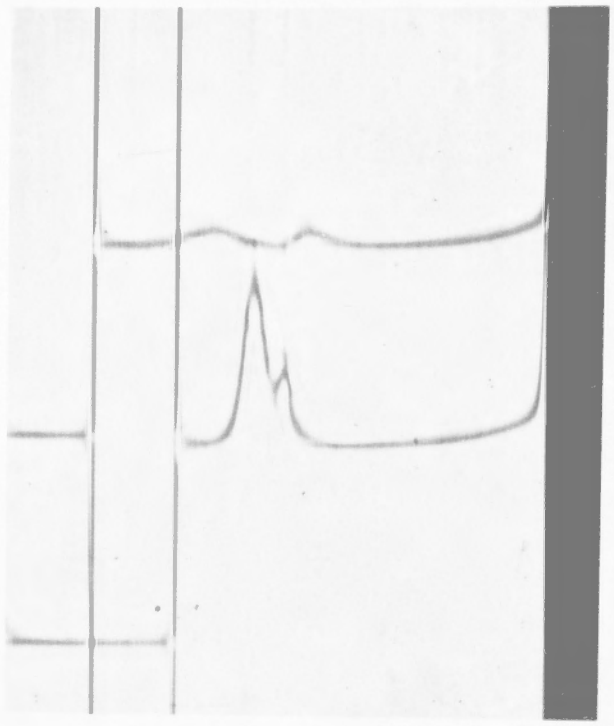


Fig. 6:4. Sedimentation velocity patterns of fraction II. Details of the preparation are given in the text. The speed was 50,740 rpm and the schlieren phase plate angle was 60°. Direction of sedimentation is from left to right. Top, 0.117 g per dl; bottom 0.229 g per dl; 20 min after reaching speed.

It is clear that two major fractions have resulted. Fraction 11 with a density greater than 1.65 g per ml and fraction 111 with a density less than 1.55 g per ml. The greater part of the uronic acid containing material was contained in fraction 11. Fraction 111 consisted largely of protein. A preparative experiment was subsequently performed. Each tube was cut into three fractions as indicated in fig 6:3 and similar fractions from several tubes were pooled, dialysed free of CsCl, concentrated by ultrafiltration and the properties of these two fractions were determined.

6:2. EXAMINATION OF FRACTION 11.

Velocity sedimentation of fraction 11 showed the presence of two sedimenting boundaries (fig 6:4), similar to those observed with the original proteinpolysaccharide material (fraction 1). The concentration dependences of the sedimentation coefficients of the components of fraction 11 are included in fig 6:2A, B to allow comparison with fraction 1; the values agree closely.

The results of this experiment show that nasal cartilage proteinpolysaccharide material is density heterogeneous, however density gradient centrifugation is not instrumental in effecting a separation of the materials that constitute the two boundaries observed in velocity sedimentation. The results do suggest however

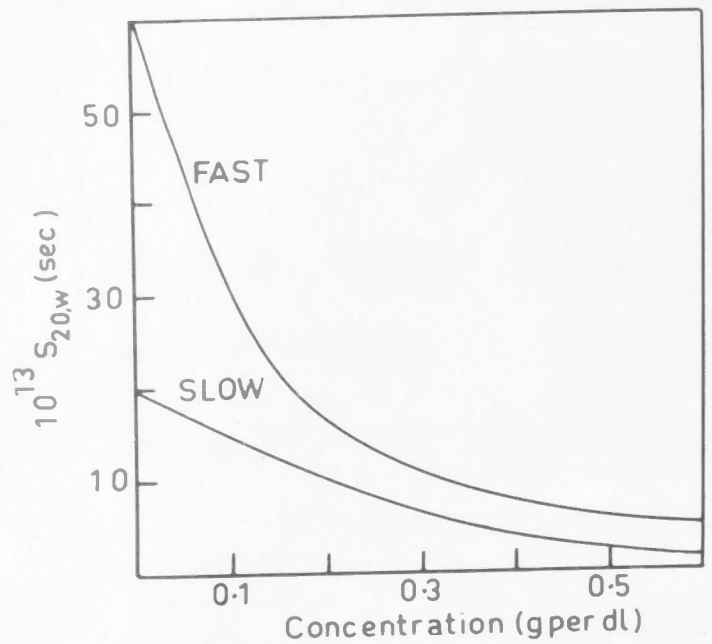


Fig. 6:5. The effect of total concentration of the proteinpolysaccharide on the fast and slow sedimenting components. Note that below approximately 0.06 g per dl there is a significant difference between the sedimentation coefficients of the fast and slow components.

that little or no degradation of these components has occurred during the density gradient centrifugation process. Chemical analyses of fraction 11 are included in table 6:1.

6:2:1. SEPARATION OF THE COMPONENTS OF FRACTIONS 1 AND 11.

It has been shown that the concentration dependences of the fast and slow components present in fractions 1 and 11 are similar. Further, it can be estimated from fig 6:2 A, B that the fast components have sedimentation coefficients which extrapolate to approximatley 70 S while the slow components have sedimentation coefficients extrapolating to approximately 20 S. Fig 6:5 shows more clearly the differences which develop between the sedimentation coefficients of these two fractions as the concentration decreases. It can be seen that below about 0.06 g per dl the difference has become quite appreciable. In view of these observations it seemed feasible that a separation of these two components was possible by means of rate-zonal centrifugation.

6:2:2. RATE ZONAL CENTRIFUGATION.

Rate zonal centrifugation has been referred to previously, section 4:1:1. The separation depends, in part, upon the differences between the sedimentation coefficients of the various components present in the system.

Table 6:1. Chemical Analysis of Proteinpolysaccharide Fractions from Bovine Nasal Cartilage.

ANALYSIS	FRACTION I	II	IA	IIA	IB	IIB
Uronic acid	24.0	24.5	19.9	21.9	26.0	24.0
Hexosamine	24.8	24.7	19.9	20.3	24.8	22.8
Glucosamine	3.2	3.4	3.0	1.8	1.8	4.8
Galactosamine	21.6	21.3	16.9	18.5	23.0	18.0
Hexose[a]	5.4	5.5	8.9	7.0	3.5	2.4
Sulphate[b]	13.7	12.4	11.4	11.4	13.8	12.2
Polypeptide[c]	29.3	17.6	21.8	16.2	12.7	6.8
Polypeptide[d]	19.7	13.8	20.2	17.1	12.0	6.9
Molar ratio. Galactosamine = 1.00						
Uronic acid	1.02	1.06	1.08	1.09	1.04	1.22
Hexosamine	1.15	1.16	1.18	1.10	1.08	1.27
Glucosamine	0.15	0.16	0.18	0.10	0.08	0.27
Galactosamine	1.00	1.00	1.00	1.00	1.00	1.00
Hexose	0.25	0.26	0.52	0.38	0.15	0.13
Sulphate	1.18	1.08	1.26	1.14	1.11	1.26

[a], as galactose; [b], as SO_4^{2-}; [c], determined by the method of Lowry *et al*. (1951); [d], calculated from the amino acid analysis.

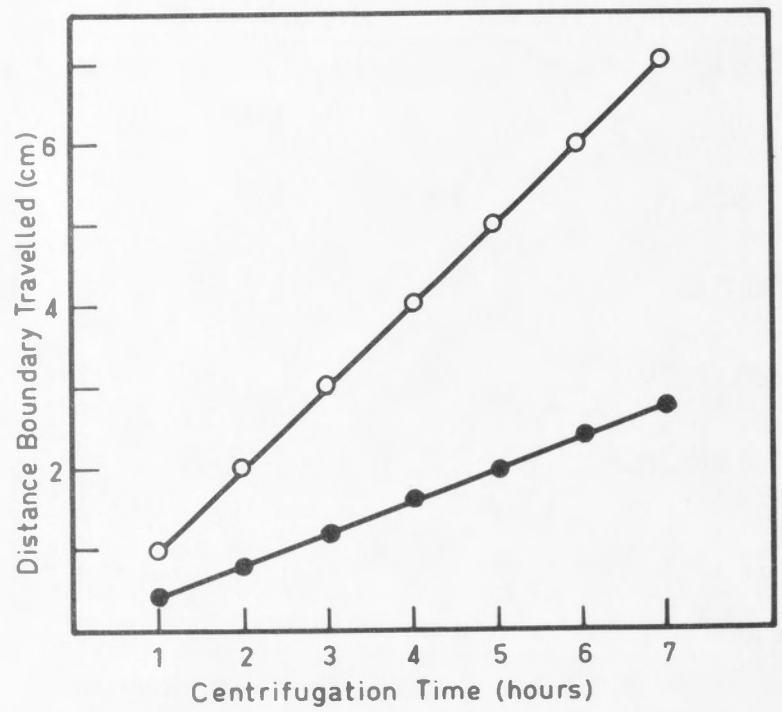


Fig. 6:6. The predicted difference in the distances travelled by the boundary constituting the fast component, 0——0, and the boundary constituting the slow component, ●——●, when a solution of the proteinpolysaccharide, fraction I, is centrifuged for varying periods in the SW 25.2 swing-out rotor at 25,000 rpm. Initial total concentration was assumed as 0.06 g per dl.

In considering a preparative rate zonal centrifugation for the separation of the components of fractions 1 and 11 the Beckman-Spinco swing-out bucket rotor no. SW 25.2 was thought to be optimal since this rotor is capable of accommodating large sample volumes; the maximum attainable speed of this rotor is not high, but, in this instance, high angular velocities are not required.

The sedimentation coefficient has been defined previously, section 2:3:1, equation 2:1. It is possible from this definition to calculate the rate of movement of the boundaries through the medium during centrifugation.

In the case of the SW 25.2 rotor the maximum allowable speed is 25,000 rpm and the distance of the meniscus from the axis of rotation is of the order of 8 cm. Assuming that the sedimentation coefficient of the fast component at a concentration of 0.06 g per dl is 50×10^{-13} sec and that of the slow component 20×10^{-13} sec, the distance travelled by the respective components with respect to time can be calculated. The results of such a computation are illustrated in fig 6:6. It can be seen that for centrifugation runs above three hours at 25,000 rpm there is a significant difference in the distances transversed by the two components, indicating that a separation of the components should be possible using centrifugation times greater than three hours. However,

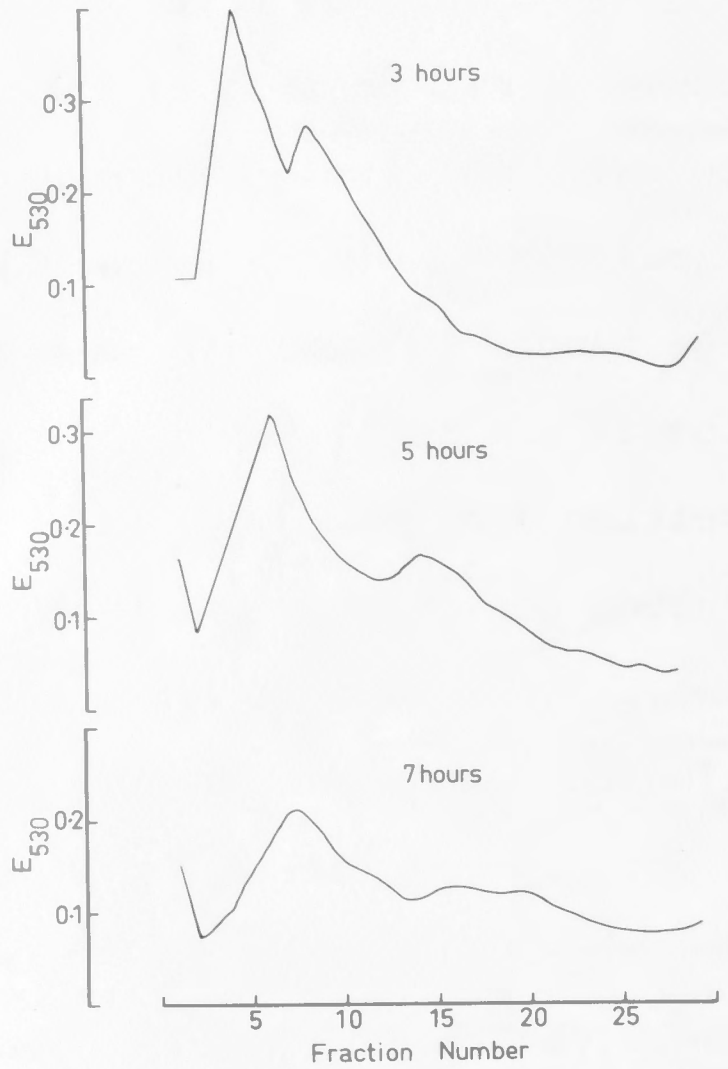


Fig. 6:7. The distribution of uronic acid after rate zonal centrifugation of fraction 1. Centrifugation was performed in the swing-out SW 25.2 rotor in a Beckman model L2 ultra - centrifuge at 25,000 rpm for periods of 3, 5 and 7 hours. NaCl gradients, total volume 55 ml, range 5 - 30 percent (w/v) were used.

the marked concentration dependences of the sedimentation coefficients would cause the boundaries to spread significantly (Vinograd and Bruner, 1966). Thus in order to determine the most suitable conditions for separation, an experimental approach must be used, using the calculations as a guide.

Solutions of nasal cartilage proteinpolysaccharide (fraction 1) were centrifuged in NaCl gradients for three, five and seven hours in the model L2 ultracentrifuge using the SW 25.2 rotor at 25,000 rpm. The temperature was controlled at 5°.

Linear gradients of NaCl were prepared by successively layering under each other, NaCl solutions of increasing concentrations (total volume 55 ml; range of NaCl concentrations 5 to 30 g per 100 ml). The gradients were stored at 4° for 24 hours before use. A solution of the proteinpolysaccharide (2.5 ml containing approximately 1.5 mg) was carefully layered on top of the gradient and centrifugation was commenced without delay.

After centrifugation 2-ml fractions were collected dropwise from a small hole made in the bottom of the tube. The uronic acid content of each fraction was determined. The distribution of uronic acid after the solutions had been centrifuged is illustrated in fig 6:7. It can be seen that after three hours centrifugation the boundaries

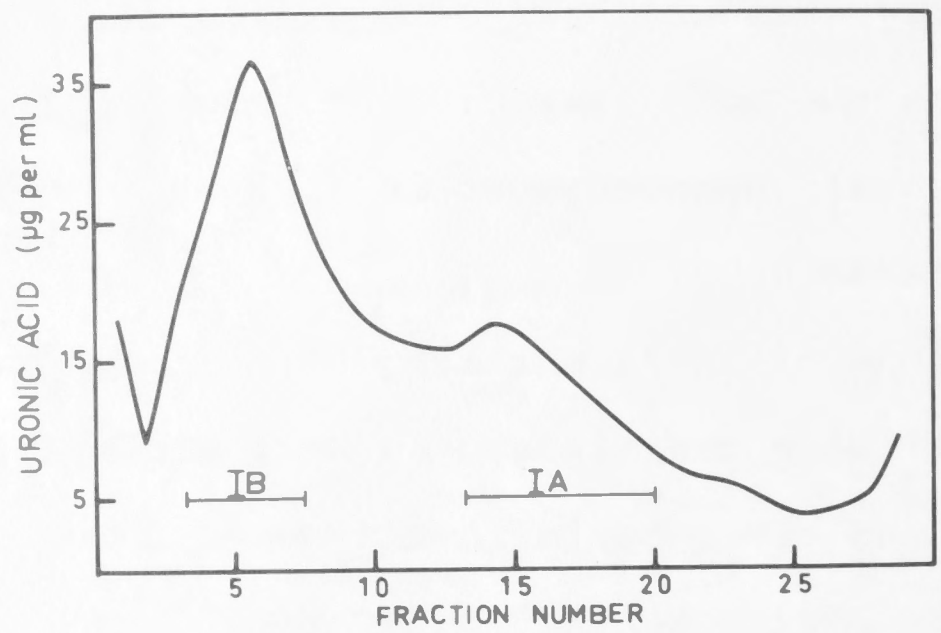


Fig. 6:8. Distribution of uronic acid after rate zonal centrifugation of a solution of fraction 1. Centrifugation was performed in the swing-out SW 25.2 rotor in the Beckman model L2 ultra - centrifuge at 25,000 rpm for 5 hours. NaCl gradients, total volume 55 ml, range 5 - 30 percent (w/v) were used. Fractions IA and IB were obtained as indicated. Centrifugation of fraction II gave the preparation IIA and IIB.

are well formed but are close. After five hours there is a better separation of the peaks but the boundaries have begun to spread, while after seven the spreading has increased to such an extent that the boundary constituting the fast component is hardly discernable. It therefore appeared that a centrifugation time of five hours offered the best conditions for effecting a separation.

A preparative experiment was carried out using the SW 25.2 rotor at 25,000 rpm. The centrifugation time was five hours. The distribution of uronic acid-containing material obtained in the manner described above is illustrated in fig 6:8. Exactly the same distribution was obtained for fraction 11 as for fraction 1. The distribution of uronic acid along the tube while indicating the presence of the two main sedimenting boundaries also shows that a complete separation of the two components has not been obtained. However fractions comprising the two uronic acid peaks were pooled as shown in fig 6:8. The pooled fractions were then dialysed free of NaCl, concentrated by ultrafiltration and then examined in the analytical ultracentrifuge. The fast fractions were designated 1A and 11A depending upon whether they were obtained from fraction 1 or fraction 11 and similarly the slow fractions 1B and 11B.

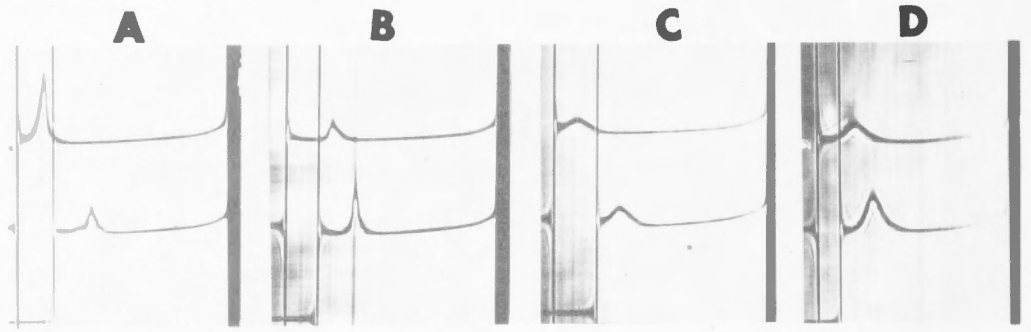


Fig. 6:9. Sedimentation velocity patterns of proteinpolysaccharide fractions obtained after rate zonal sedimentation. The speed in all cases was 50,740 rpm and the schlieren phase plate angle 60°. Experiments were carried out in the standard buffer. A, fraction IA: top 0.085 g per dl; bottom 0.053 g per dl; 8 min after reaching speed. B, fraction IIA; top 0.053 g per dl; bottom 0.073 g per dl; 6 min after reaching speed. C, fraction IB: top 0.037 g per dl; bottom 0.063 g per dl; 14 min after reaching speed. D, fraction IIB: top, 0.085 g per dl; bottom, 0.177 g per dl; 39 min after reaching speed.

Typical velocity sedimentation patterns of these separated components are shown in fig 6:9A, B, C and D. It can be seen that the isolated materials sedimented as single boundaries in the analytical ultracentrifuge.

Analytical data for these several fractions are included in table 6:1. to allow comparison with fractions 1 and 11. Fractions 1, 1A and 1B contain respectively more protein than the corresponding fractions 11, 11A and 11B which were obtained from the density gradient separation. Otherwise fraction 1 and 11, 1A and 11A and 1B and 11B are,respectively,very similar. The amino acid profile for each of these fractions is given in table 6:2. There is a close similarity in all cases. Also the profile is similar to that obtained by Pal et al. (1966) for a similar preparation from nasal cartilage.

6:3. EXAMINATION OF FRACTION 111. *

Fraction 111 was obtained from the low density region after a solution of crude proteinpolysaccharide from bovine nasal cartilage had been centrifuged to equilibrium in a CsCl gradient of initial density 1.63 g per ml. It can be seen from fig 6:3 that fraction 111 consists largely of protein.

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* The investigations of fraction 111 were mainly the work of Dr. J.R.Dunstone. They have been included in this thesis for completeness in the analysis of cartilage proteinpolysaccharides and also so that they might be compared with similar fractions obtained from porcine aorta.

Table 6:2. Amino acid composition of uronic acid-containing fractions[a]

Amino acid	Fraction I	Fraction IA	Fraction IB	Fraction II	Fraction IIA	Fraction IIB	Chondro-mucoprotein[b]
Lysine	4.2	4.1	3.9	3.6	3.6	3.9	3.5
Histidine	1.7	1.8	2.1	1.9	1.6	0.6	1.7
Arginine	5.3	5.2	4.5	5.0	5.5	3.4	4.5
Aspartic acid	9.2	9.3	8.6	8.2	8.8	7.8	8.5
Threonine	5.5	5.5	5.4	5.6	5.7	5.8	5.8
Serine	7.3	7.1	9.1	8.0	7.9	13.4	9.2
Glutamic acid	12.3	12.4	12.5	12.3	13.0	14.7	12.5
Proline	9.4	8.4	9.1	9.5	9.2	6.9	9.3
Glycine	10.7	8.7	10.6	10.1	9.8	15.0	12.7
Alanine	7.5	6.9	6.7	7.6	7.6	7.0	7.4
Half cystine	0.9	1.6	0.8	0.8	0.8	-	0.7
Valine	6.9	7.1	6.7	7.2	6.7	5.9	6.3
Methionine	1.1	1.2	1.0	1.1	0.8	0.6	0.7
Isoleucine	3.7	4.5	4.0	4.0	3.9	3.7	3.8
Leucine	8.2	8.9	8.8	8.2	7.8	7.8	7.8
Tyrosine	2.5	3.1	2.4	2.7	3.0	0.5	1.9
Phenylalanine	3.6	4.2	3.8	4.2	4.3	3.0	3.5

[a] All values are expressed as μmoles amino acid per 100 μmoles of amino acids estimated. No corrections have been applied for the degradation of certain amino acids that occurs during acid hydrolysis in the presence of carbohydrate.

[b] Calculated from values of Pal et al. (1966)

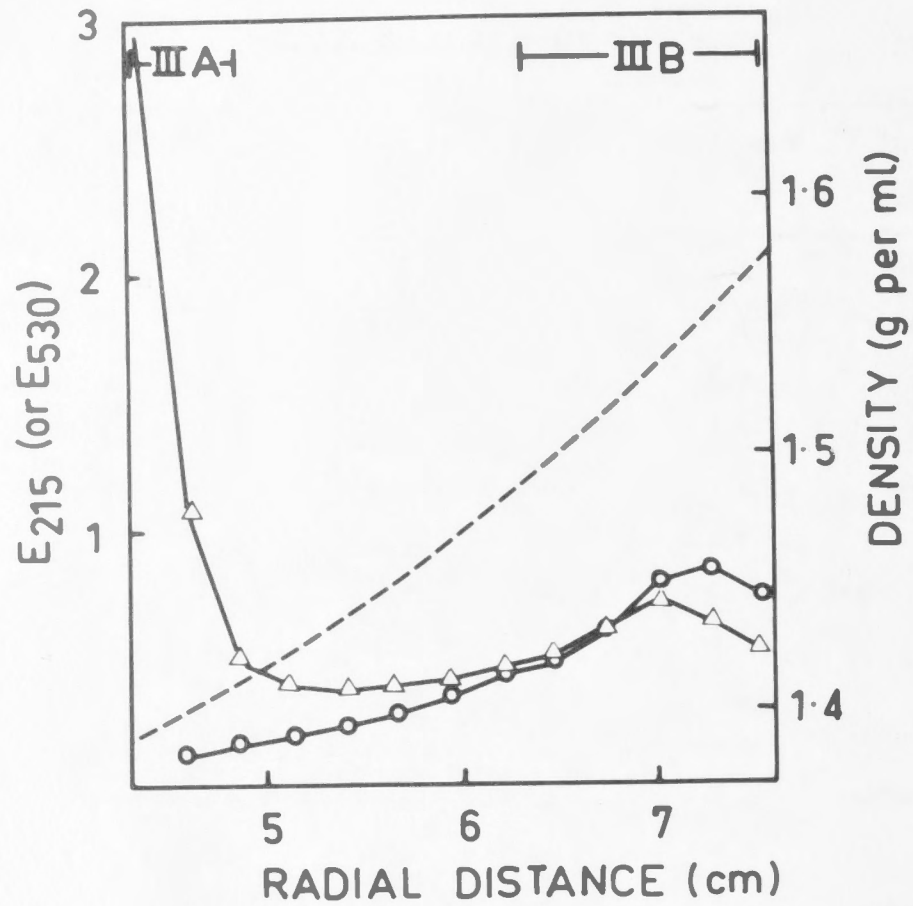

Fig. 6:10. Distribution of uronic acid and protein after centrifuging fraction III to equilibrium in a CsCl density gradient. The initial density was 1.47 g per ml. The density is shown by the line – – – –; uronic acid by O————O; and protein by Δ————Δ. Sedimentation was continued for 48 hours in the No. 40 rotor of a Beckman model L ultracentrifuge. Fractions IIIA and IIIB were obtained as illustrated.

6:3:1. DENSITY GRADIENT CENTRIFUGATION OF FRACTION 111.

Fraction 111 has a density less than 1.55 g per ml. This material has reached equilibrium in a similar density range to that of the glycoprotein components of aorta. Thus like the aortic material a solution of fraction 111 was recentrifuged to equilibrium in a CsCl density gradient of lower initial density.

An aliquot of fraction 111 was adjusted to an initial density of 1.47 g per ml and centrifuged to equilibrium in the preparative ultracentrifuge (no. 40 rotor). The results of this experiment are illustrated in fig 6:10. The final density range was 1.38 to 1.58 g per ml. It can be seen that there has been a separation of fraction 111 into two further fractions, the less dense fraction (fraction 111A) with an apparent density less than 1.4 g per ml and a denser fraction, 111B, with an apparent density of 1.53 to 1.55 g per ml. The tubes were cut into three sections as indicated in fig 6:10 and identical fractions from several tubes were pooled, dialysed free of CsCl, concentrated by ultrafiltration and chemically analysed.

6:3:1:1. Examination of Fractions 111A and 111B.

The absence of material similar to fraction 111A from fraction 111B was demonstrated by polyacrylamide gel electrophoresis (fig 6:11). The electrophoretic

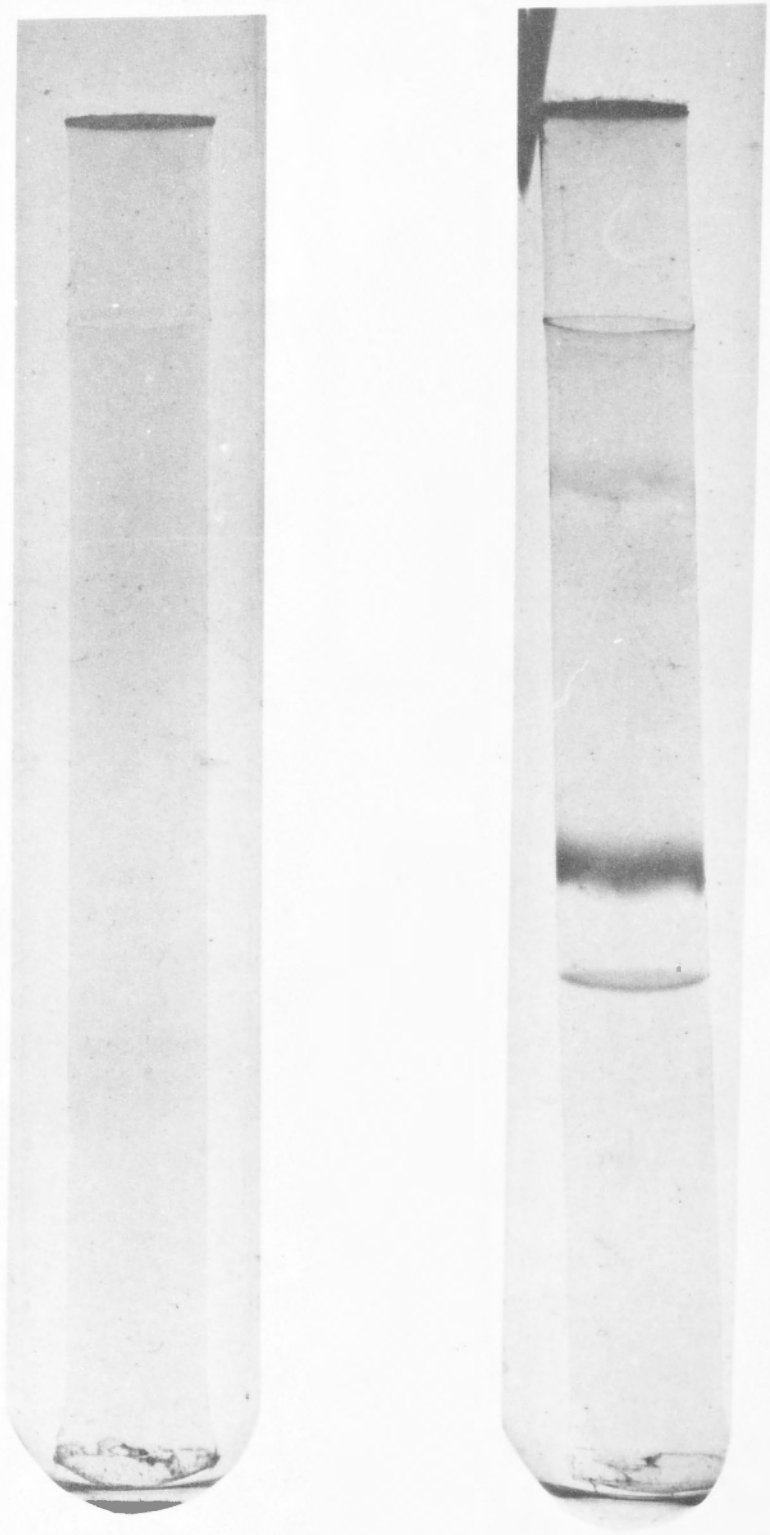

Fig. 6:11. Electrophoresis on polyacrylamide gel of fraction IIIA and IIIB.

patterns show the presence of a major component and two minor components in fraction 111A. No corresponding material was detected in the gel columns when samples of fraction 111B were examined; in fact no material entered the gel. However fraction 111B showed multiple sedimenting boundaries during velocity sedimentation; at concentrations near 0.1 g per dl this material contained 14 S and 19 S components.

Some analytical data for fraction IIIB are given in table 6:3. There is a nearly equimolar proportion of uronic acid and galactosamine and also a relatively large amount of hexose. Because only very small amounts of material were available only a preliminary analysis could be made on fraction 111A. The results of this analysis indicated that this material contained small amounts of uronic acid, glucosamine and galactosamine and significantly higher amounts of hexose. Paper chromato - graphy of an acid hydrolysate of fraction 111A (fig 6:12 A) indicated that galactose and glucose were present. Electrophoresis of fraction 111A on polyacrylamide gel showed that a large proportion of the material was contained in one band (fig 6:12 B). It would therefore appear that fraction 111A is a glycoprotein or a mixture of glycoproteins.

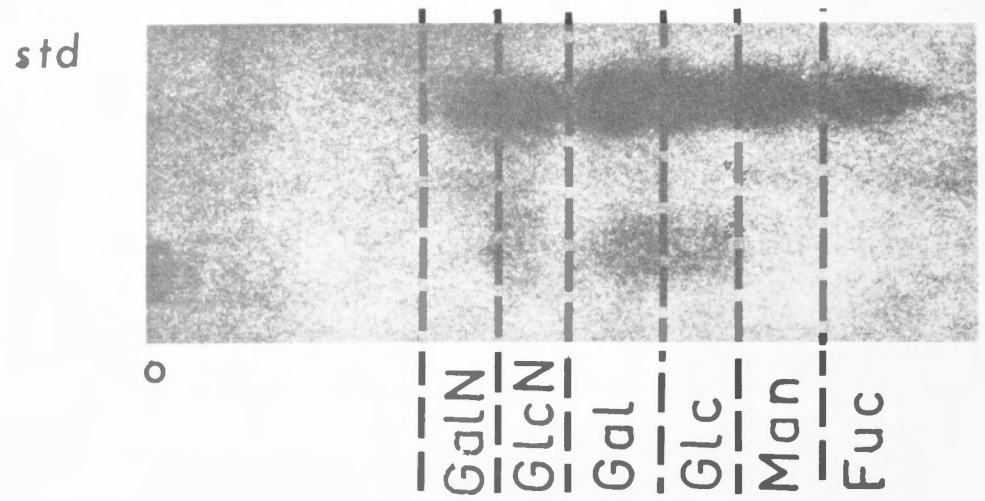

Fig. 6:12:A. Paper chromatography of fraction IIIA. Hydrolysis - 7 h in 2N HCl at 100°. Solvent - Butanol: pyridine : water (6:4:3). Stain - Analine hydrogen phthalate.

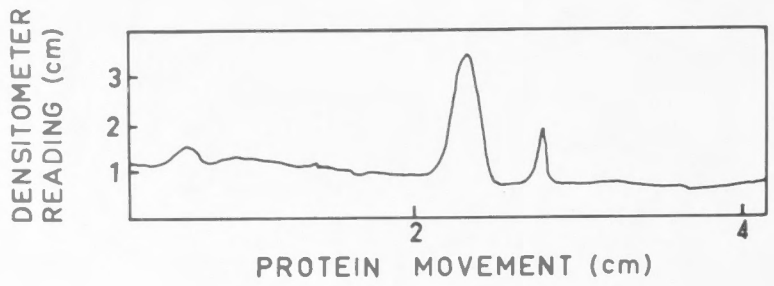

Fig. 6:12:B. Polyacrylamide gel electrophoresis of fraction IIIA. The stained column was photographed, and the negative measured on a Beckman-Spinco Analytrol film densitometer.

The amino acid composition of fractions 111A and 111B are given in table 6:4. Since fraction 111A appears to be a glycoprotein, the analysis of fraction G1, another glycoprotein that was obtained from aorta (chapter 5, section 5:1:3 is included for comparison.

Table 6:3. Chemical Analysis of Fraction 111B.

ANALYSIS	percentage	Molar ratio[a]
Uronic acid	10.2	1.06
Hexosamine	10.5	1.19
Glucosamine	1.7	0.19
Galactosamine	8.8	1.00
Hexose[b]	7.1	0.80
Sulphate	5.6	1.20
Polypeptide[c]	39.3	-
Polypeptide[d]	35.6	-

[a], Galactosamine value taken as 1.00; [b], as galactose,
[c], determined by the method of Lowry et al. (1951);
[d], calculated from the amino acid analysis.

Fraction G1 was prepared under the same experimental conditions as fraction 111A. It can be seen that the amino acid profiles are very similar. They both have increased amounts of the basic amino acid lysine when compared with the amino acid profiles of the glycosamino-

Table 6:4. Amino Acid Analyses of Fractions IIIA and IIIB.

Amino Acid	µmoles per 100 µmoles of amino acid determined		
	IIIA	GI	IIIB
Lys	9.6	9.6	5.0
His	2.7	2.2	1.9
Arg	4.5	4.9	5.2
Asp	9.4	10.1	9.4
Thr	5.8	5.5	4.6
Ser	5.5	6.5	6.6
Glu	13.8	13.9	11.6
Pro	5.6	5.4	9.3
Gly	6.9	6.6	14.7
Ala	8.7	8.8	7.7
CyS	1.1	2.2	0.3
Val	6.9	6.5	5.6
Met	0.6	1.3	1.1
Ile	2.4	4.1	3.5
Leu	9.7	8.0	7.7
Tyr	2.4	0.9	2.4
Phe	4.4	3.3	3.4

glycuronoglycan protein components, for example fraction 111B. This latter fraction has an amino acid composition similar to the glycosaminoglycuronoglycan proteins of fraction 11, see table 6:2.

It would thus appear that the material of low density isolated from nasal cartilage by density gradient centrifugation contains a glycoprotein fraction and a glycosaminoglycuronoglycan protein fraction. Dr.J.R. Dunstone was able to obtain a similar fractionation of the components of fraction 111 by gel filtration on Sephadex G 200 (Dunstone and Franek, 1967). The chemical composition of the components isolated in this way were similar in composition to the components isolated by density gradient centrifugation.

6:4. CONCLUDING REMARKS.

Recent evidence (Pal et al., 1966) suggests that the proteinpolysaccharide extracted from bovine nasal cartilage consists of a series of compounds which differ not only in chemical composition but also with regard to their physical properties. This investigation has utilized these differing physical properties to effect a separation of the components.

Previous workers have reported ultracentrifugal heterogeneity under conditions of low concentration and high ionic strength (Webber and Bayley, 1956; Pal et al.,

1966). In this work ultracentrifugal heterogeneity was shown to be observed over a wide range of concentration and at low ionic strengths. At concentrations below 0.06 g per dl the sedimentation coefficients of the boundaries were sufficiently different to allow a separation of the two components by rate zonal centrifug - ation.

Luscombe and Phelps (1967a) have reported that their preparation of nasal cartilage proteinpolysaccharide was homogeneous although polydisperse when examined in CsCl or sucrose density gradients. The experimental results presented here show that this was not the case with our preparations which had been prepared similarly. When fraction 1 was centrifuged in a CsCl gradient two fractions resulted; (fractions 11 and 111). Fraction 11 contained two glycosaminoglycuronoglycan components while fraction 111 contained glycosaminoglycuronoglycan components and glycoprotein material.

The two glycosaminoglycuronoglycan proteins of fraction 11 could be further fractionated by rate zonal centrifugation and were found to be similar to the corresponding preparations obtained from fraction 1. Further, since these isolated components (i.e. fractions 1A, 11A, 1B and 11B) always sedimented in the ultracentri- fuge as single boundaries and since the $s_{20,w}$ values were

in close agreement with values predicted from the unseparated materials, a further physico-chemical investigation of these fractions was undertaken.

In table 6:5 the yields of these various fractions obtained from one gram of dried cartilage have been summarized. The yield of fraction 1 (24 percent) from a single extraction represents about two thirds of the total water-extractable proteinpolysaccharide (Pal et al., 1966). A second water extraction accounts for the remaining extractable material, which is chemically similar to that obtained in the first extraction.

Table 6:5. Yields of the various Proteinpolysaccharide Fractions obtained from Bovine Nasal Cartilage.

FRACTION	AMOUNT OF DRIED CARTILAGE g per g
1	0.24
1A	0.06
1B	0.10
11	0.16
11A	0.04
11B	0.06
111	0.02

Chapter 7.

Molecular weight and Particle Shape.

7:1. INTRODUCTION

The molecular weight and particle shape of macromolecules may be established from appropriate physical measurements on very dilute solutions. The various physical methods in use at present involve measurements respectively, of osmotic pressure, light scattering, sedimentation equilibrium and sedimentation velocity in conjunction with diffusion or solution viscosity. All except the last mentioned are absolute methods for determining molecular weight. Each requires extrapolation to infinite dilution for rigorous fulfilment of the requirements of theory. These various physical methods depend basically on evaluation of thermodynamic properties of the solutions (i.e., the change in the free energy due to the presence of the polymer molecule) or the kinetic behaviour (i.e. frictional coefficient or viscosity increment), or a combination of the two.

The theories capable of describing the behaviour of the solute molecules are presently limited to a number of model objects, namely, spheroids, cylindrical rods and random coils. Thus the use of these theories can lead only to values which apply to the model used, not to the real particle; their relevance to the real particle will depend upon how closely the model resembles

it.

In the work to be presented in this chapter, molecular weight estimations and particle shapes have been inferred only from sedimentation velocity and viscosity data.

7:1:1. MOLECULAR WEIGHT.

The equation used in determining the molecular weight from sedimentation velocity and viscosity data is derived primarily from hydrodynamic considerations. Its use requires a knowledge of the "equivalent hydrodynamic ellipsoid". This is a hypothetical, rigid, impermeable ellipsoid which would behave, when it undergoes rectilinear or rotational motion in a fluid, as the molecule under study is observed to behave. Its volume V' and axial ratio J, are both related to the observed parameters through the Perrin (1936) functions and the Simha (1940) viscosity functions as follows: According to Perrin (1936) the frictional coefficient of the effective hydrodynamic ellipsoid is given by the equation

$$\frac{f}{f_o} = \frac{1}{F} \quad \ldots\ldots\ldots\ldots\ldots \quad (7.1)$$

where f is the mean frictional coefficient at infinite dilution and f_o is the frictional coefficient of a

sphere of radius r, having the same volume as the equivalent hydrodynamic ellipsoid. Since $f_o = 6\pi\eta_o r$ where where $r = (3/4\pi)^{1/3}(V')^{1/3}$ then

$$f_o = 6\pi\eta_o(3/4\pi)^{1/3}(V')^{1/3} \quad \ldots\ldots\ldots \quad (7.2)$$

where η_o is the viscosity of the solvent and

$$f = \frac{6\pi\eta_o(3/4\pi)^{1/3}(V')^{1/3}}{F} \quad \ldots\ldots\ldots \quad (7.3)$$

The Svedberg equation for the sedimentation coefficient at infinite dilution (Svedberg and Pederson, 1940) is

$$s_o = \frac{M(1 - \bar{v}\rho)}{Nf} \quad \ldots\ldots\ldots\ldots\ldots \quad (7.4)$$

where M is the molecular weight, $\bar{v}$ the partial specific volume of the solute and ρ the density of the solvent. The intrinsic viscosity $[\eta]$ is related to the volume of the effective hydrodynamic ellipsoid (Scheraga and Mandelkern, 1953) i.e.

$$V' = \frac{[\eta]100\ M}{N\nu} \quad \ldots\ldots\ldots\ldots\ldots \quad (7.5)$$

where ν is the shape factor which depends on the axial ratio J of the effective hydrodynamic ellipsoid. Thus combining equations 7.1, 7.3, 7.4 and 7.5 and rearranging

$$\frac{Ns_o[\eta]^{1/3}\eta_o}{M^{2/3}(1 - \bar{v}\rho)} = \frac{\nu N}{16200\pi^2} \cdot \frac{f_o}{f} \quad . . . . (7.6)$$

the quantity on the right hand side of equation 7.6 has been denoted β by Scheraga and Mendelkern (1953), thus equation 7.6 can be rewritten

$$M^{2/3} = \frac{Ns_o[\eta]^{1/3}\eta_o}{\beta(1 - \bar{v}\rho)} \quad . . . . . (7.7)$$

where β is called the Scheraga-Mandelkern shape function and is a function of shape alone. Thus in order to calculate the molecular weight from sedimentation velocity and viscosity data alone, some prior knowledge as to the shape of the molecule in terms of the equivalent hydrodynamic ellipsoid is required. Ogston (1953) has given some useful estimations whereby the dimensions of molecules can be obtained from sedimentation velocity and viscosity data. The application of such data is based on the theory of flow through porous plugs (Sullivan and Hertel, 1942). Ogston (1953) has utilized the coefficient of concentration dependence K_s, of the reciprocal sedimentation coefficient i.e.

$$\frac{1}{s} = \frac{1}{s_o}(1 + K_s c) \quad . . . . . (7.8)$$

to establish a relationship between the intrinsic viscosity and J the axial ratio (i.e. a/b, the ratio of

the semi-axis of revolution to the equatorial semi-axis). The relationship is given in the form

$$\frac{\zeta}{k}\ \frac{s_o d(1/s)/dc}{[\eta]} = \frac{\chi^2(J)}{\xi(J)\psi(J)} \quad . . . . . \quad (7.9a)$$

(equation 13, Ogston, 1953)

$$\frac{\zeta}{k}\ \frac{K_s}{[\eta]} = \frac{\chi^2(J)}{\xi(J)\psi(J)} \quad . . . . . \quad (7.9b)$$

where ζ and k are constants and $\chi^2(J)$, $\xi(J)$ and $\psi(J)$ are functions of J. In a collected table Ogston (1953) has listed values for the functions of J and combinations of these various functions, including the β function, over the range J equals 100 to J equals 0.01, (a copy of this table is seen in appendix 2). By determining K_s and $[\eta]$ from experimental data and coupling with suitable values of ζ and k values for $\chi^2(J)/\xi(J)\psi(J)$ are obtained; reference to the table of Ogston (1953) provides a value of J and the corresponding value of β. By substituting this value of β into equation 7.7 an estimate of the molecular weight can be obtained.

7:1:2. PARTICLE SHAPE.

In an attempt to establish some generalizations Creeth and Knight (1965) have critically reviewed the current theories available for the interpretation of sedimentation and viscosity data in the estimation of

molecular shape.

These authors considered that use of the Scheraga-Mendelkern shape factor β, in estimating particle shape is limited because the value of β varies only very slowly with axial ratio, so that extremely high accuracy in the determination of s, $[\eta]$, M and $\bar{v}$ is necessary if the function is to be interpreted in this way. A further difficulty arises in that β has a characteristic value for a random coil configurations identical with that of a rod of axial ratio 15. The authors therefore directed their attention to the ratio $K_s/[\eta]$.

A quantitative theory relating K_s to molecular parameters was developed by Fessler and Ogston (1951); Ogston (1953) has suggested its use to determine J and V' (the hydrodynamic volume per gram of unsolvated solute). However the function of J relating K_s to J, i.e. $\chi^2(J)/\xi(J)\psi(J)$ has the same disadvantages as the β function; it varies only slowly with the axial ratio. Further, uncertainty also exists in assigning correct values to the constants ζ and k (equation 7.9b). A value of 2/3 for ζ seems to be reasonable (Ogston, 1953) but the selection of a proper value for k presents a more difficult problem; values of 1.35, 1.8 and 3.0

have been used but it has been suggested that k may vary with molecular shape (Ogston, 1953, 1961).

Wales and Van Holde (1954) have shown that for random-coiled polymers $K_s/[\eta]$ was approximately constant and equal to about 1.6. Wales and Van Holde employed Burgers (1942) theory for the concentration dependence of sedimentation of spherical particles and the Mandelkern-Flory (Flory, 1953) polymer theory to give a theoretical basis for this relationship and to predict a characteristic value for $K_s/[\eta]$ of 1.6.

The conclusions, reached by Creeth and Knight (1965) concerning the ratio $K_s/[\eta]$ were that in general, globular proteins or spherical proteins gave values of $K_s/[\eta]$ that were fairly high, 1.5 to 1.7, while proteins known to be assymmetric by other criteria gave markedly lower values. Denatured proteins on the other hand gave high values similar to those characteristic of spherical particles and thus resembled the synthetic coiled polymers of the type originally considered by Wales and Van Holde (1954) to have $K_s/[\eta]$ values of 1.60. For values greater than 1.7, Creeth and Knight (1965) were unable to offer any interpretation.

Ogston (1953) has concluded that estimates of particle dimensions obtainable from hydrodynamic

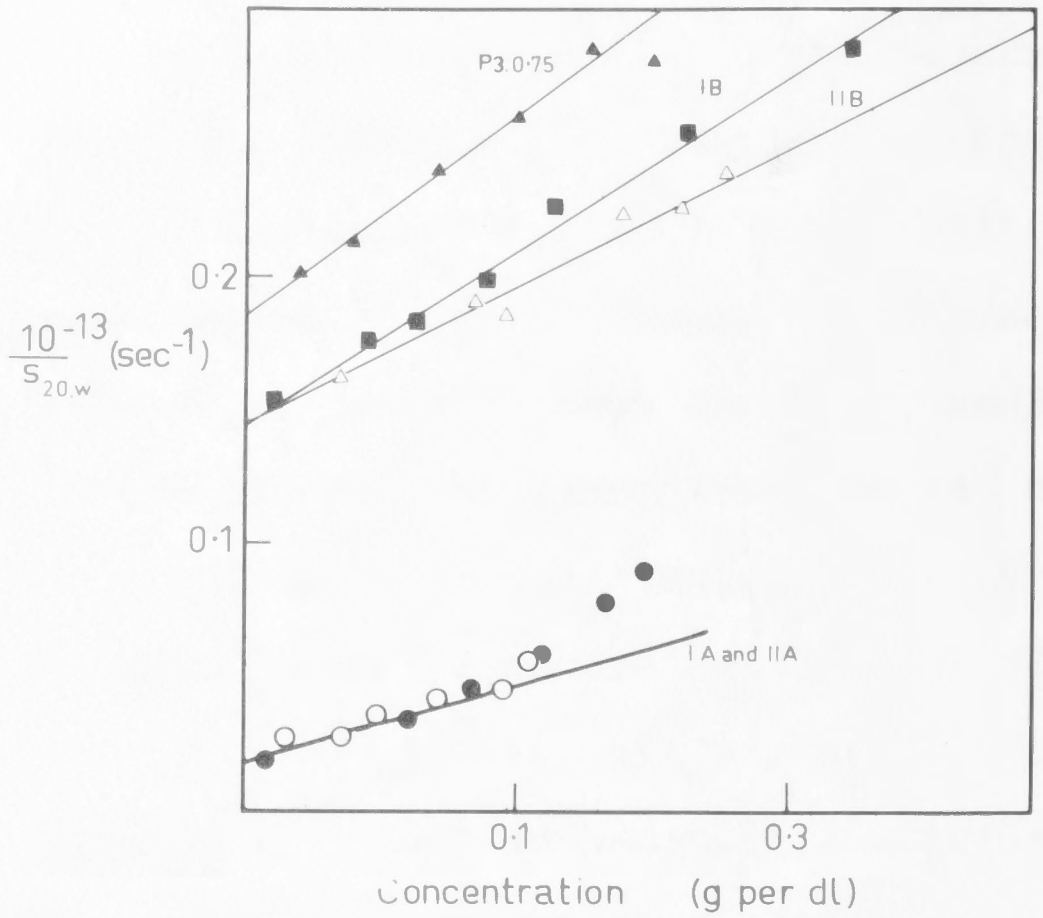

Fig. 7:1. Variation of the reciprocal of the sedimentation coefficients of the nasal cartilage and aortic proteinpolysaccharides with concentration. Standard phosphate buffer pH 6.75.
● , IA; ○ , IIA; ■ , IB; △ , IIB; ▲ , P3 0.75.

measurements are often at best semi-quantitative, nevertheless, these estimates may be helpful in forming a picture of the solute particle as it exists in solution.

7:2. EXPERIMENTAL

The sedimentation velocity and reduced viscosities of fractions IA, IIA, IB and IIB from nasal cartilage and the fraction P3.0.75 from aorta were obtained using the methods described in chapter 2, section 2:3. Measurements were made on several preparations of each fraction over a concentration range of 0.003 to 0.40 g per dl.

Fig. 7.1 illustrates the dependence of the sedimentation coefficients on concentration for these several fractions. The 1/s against c plots shown have been calculated from the experimental data by the method of least squares, however only one or two sets of experimental data have been plotted in each instance. For fraction IA and IIA the 1/s against c plots were almost identical. The value of $1/s^{o}_{20,w}$ was 0.018×10^{13} sec. Fractions IB and IIB also had similar values for $1/s^{o}_{20,w}$ i.e. 0.146×10^{13} sec and 0.156×10^{13} sec respectively. However these latter two preparations

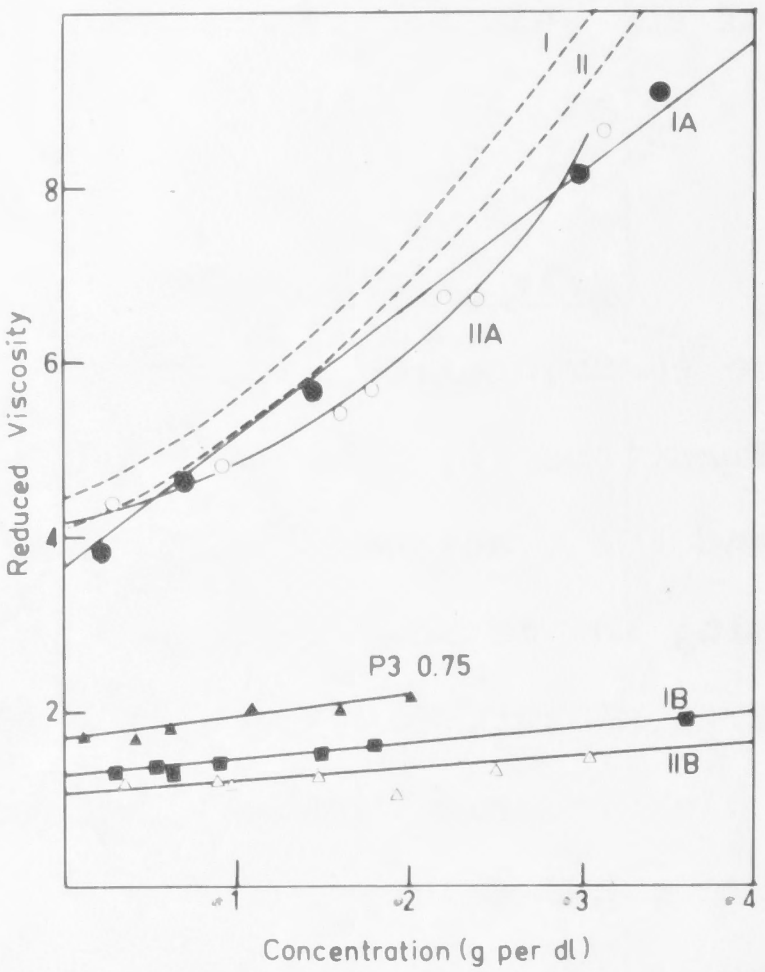

Fig.7:2. Variation of the reduced viscosity with concentration. Standard phosphate buffer pH 6.75. ● , IA; ○ , IIA; ■ , IB; △ , IIB; ▲ , P3 0.75.

showed a slightly different concentration dependence. The aortic proteinpolysaccharide had sedimentation coefficients that were smaller than the fractions IB and IIB. The value for $1/s^{o}_{20,w}$ was 0.185 x 10^{13} sec.

The concentration dependence of the viscosities of these fractions are shown in fig. 7:2. Intrinsic viscosities are given in table 7:1. Included in fig. 7:2 are the reduced viscosity against c plots for the

Table 7:1. The intrinsic viscosities and $s^{o}_{20,w}$ values for the fractions IA, IIA, IB, IIB and P3.O.75.

Fraction	$10^{13} s^{o}_{20,w}$ sec.	[n] dl per g
IA	54.0	3.7
IIA	54.0	4.1
IB	6.8	1.3
IIB	6.4	1.2
P3 0.75	5.4	1.7

fractions I and II. It can be seen that the viscosities of fractions IA and IIA has changed little from the unfractionated material. Fractions IB and IIB however have markedly lower reduced viscosities. Fraction

P3 0.75 has reduced viscosities of the same order of magnitude as fractions IB and IIB.

7:3. DISCUSSION OF EXPERIMENTAL RESULTS

The data calculated from the sedimentation velocity and viscosity experiments of the nasal cartilage protein polysaccharide fractions and the aortic protein-polysaccharide are given in table 7:2.

7:3:1. MOLECULAR WEIGHT.

The molecular weights were computed using equation 7.9 to obtain a value for J (constants included in this equation were assigned values of 1.8 and 2/3 respectively) and then by reference to the table of Ogston (1953) a value of β was found which was substituted into equation 7.7. Limitations were found in the application of this method; values of the function $\chi^2(J)/\xi(J)\psi(J)$ for fractions IA, IIA and IB were outside the range of values given in the table. However β varies little with axial ratio for values of J less than approximately 2 and the values of J for these particular preparations were considerably less than 2, thus it was assumed that a value of 2.15×10^6 could be used for β without markedly effecting the estimates of molecular weight (see appendix 2)

The two fast-sedimenting components (fractions

Table 7:2. Data calculated from the sedimentation velocity and viscosity experiments on fractions IA, IIA, IB, IIB and P3 0.75.

	FRACTION				
	IA	IIA	IB	IIB	P3 0.75
$[\eta]$	3.7	4.1	1.3	1.2	1.7
$s^{o}_{20,w}$ (S)	54.0	54.0	6.8	6.4	5.40
$\frac{d(1/s)}{dc}$	0.28	0.28	0.62	0.46	0.75
K_s	15.07	15.29	4.21	2.94	4.06
$K_s/[\eta]$	4.08	3.78	3.24	2.56	2.36
$\log \frac{\chi^2(J)^*}{\xi(J)\psi(J)}$	0.179	0.134	0.078	-0.024	-0.059
$10^{-6}\beta^*$	2.15	2.15	2.15	2.14	2.14
$10^{-6}MW$	12.02	13.02	0.32	0.28	0.26
I^*	<1/100	<1/100	<1/100	1/8	1/5

(* taken from the collected table of Ogston, 1953)

IA and IIA) had molecular weights of 12 - 13 x 10^6, while the two slow-sedimenting components (fractions IB and IIB) and the aortic proteinpolysaccharide (fraction P3 0.75) had molecular weights of approximately 0.3 x 10^6.

7:3:1:1. Fractions IA and IIA.

Many workers have suggested that preparations of proteinpolysaccharides described in the literature as having molecular weights greater than 10^6 are aggregates. Meyer (1966a, b) considers that these aggregates are composed of polymer units bridged together by basic protein. Partridge and coworkers have isolated a protein component from a preparation of nasal cartilage proteinpolysaccharides and these findings appear to be consistent with the proposal of Meyer (1966a, b).

Fractions IA and IIA both have molecular weights greater than 10^6, the only difference between these two preparations is the protein content; fraction IIA has been separated from a glycoprotein component during density gradient centrifugation. However the removal of this glycoprotein component from fraction IIA has apparently failed to alter the velocity sedimentation and viscosity characteristics of this material. Obviously a possible explanation is that the glycoprotein material

referred to here is not the specific material referred to by Meyer (1966a, b) and Partridge and coworkers (1965, 1966b). Indeed the amino acid composition differs from that of the protein and the corresponding chondroitin sulphate protein obtained by Partridge et al. (1965). The somewhat higher proportion of the basic amino acid lysine, is more in agreement with the comments of Meyer (1966a, b) concerning the nature of the protein, although high proportions of glutamic and aspartic acids are also present.

Since the protein levels of our preparations were somewhat higher than that reported by Partridge et al. (1965) it is possible that the condition used here were not sufficient to effect a dissociation of further protein from the polyanionic polymers. However if this is so, the protein must interact strongly with the polyanionic polymer since density gradient centrifugation has proved most effective in separating protein, non-sulphated polyanions and sulphated polyanions in other systems such as that described by Silpananta et al.(1967) where it was also possible to effect similar separation by chromatography on DEAE-Sephadex.

Another possibility that arises is that

fractions IA and IIA are not composed of aggregates. Such an assumption would seem feasible when it is considered that the physical and chemical properties of these fractions are almost exactly reproducible from preparation to preparation, and from solutions that have been stored for varying periods.

7:3:1:2. Fractions IB, IIB and P3.0.75.

The fractions IB, IIB and P3.0.75 all have molecular weights near 0.3×10^6. This value is of the same order of magnitude as that reported by Partridge (1966b) for a preparation of nasal cartilage proteinpolysaccharide from which a protein component has been removed by chromatography on DEAE-cellulose. Partridge (1966b) considers that 0.3×10^6 is the molecular weight of the fundamental polymer unit.

Molecular weights in the region of 0.3×10^6 have been obtained for the chondroitin sulphate-containing proteinpolysaccharides from other connective tissue sources, namely, pig laryngeal cartilage (Muir and Jacobs, 1967), human rib cartilage (Buddecke et al., 1963) and synovial fluid (Silpananta et al., 1967). However the protein content of these several preparations is very different, see table 7.3.

Table 7:3. Molecular weight and percentage protein of chondroitin sulphate-containing protein-polysaccharides from various connective tissues.

Connective Tissue	10^{-6} MW	Percent Protein
Pig laryngeal cartilage	0.23	2.0
Human rib cartilage	0.18	25.0
Synovial fluid	0.25	15.8
Bovine nasal cartilage	0.24	7.0
Fraction P3 0.75	0.26	12.0
Fraction IB	0.32	12.0
Fraction IIB	0.28	7.0

If a value of approximately 0.3×10^6 is the molecular weight of the basic unit of these protein-polysaccharides then it would appear that the fundamental units isolated from different connective tissues are characterized by differing amounts of protein core material. Such differences could perhaps alter the configuration of the macromolecules. It would seem desirable therefore that some study be made in the future on a comparison of the physical properties of these different materials which have approximately the

same molecular weight.

7:3:1:3. Fractions IA, IIA, IB, IIB and P3 0.75.

If particles of molecular weight greater than 10^6 are in fact aggregates then it would seem probable that the fundamental unit has a molecular weight in the region of 0.25 to 0.3 x 10^6, since species of this particle weight can be obtained from a number of different connective tissues. However if it is acknowledged that fractions IB, IIB and P3.0.75 are examples of the fundamental polymer unit this infers that fractions IA and IIA must be aggregates. However for the reasons mentioned previously it would seem unlikely that the aggregation occurs in the manner speculated by Meyer (1966a, b). It would be more reasonable to assume that the glycoprotein material had only been trapped within the chains of the very large macromolecules and plays no part in the ordered structure of the system. Further evidence to support this statement can be obtained by considering the fraction IB. This fraction still contains a glycoprotein component but has a similar molecular weight to the fraction IIB which has had the glycoprotein removed. Thus if aggregation was to result from the presence of a protein of this type it would be reasonable to assume

that fraction IB would have a higher molecular weight than fraction IIB which it does not.

No evidence has been produced to suggest that these fast-sedimenting components are in fact composed of aggregates. However it cannot be said with certainty that these species are discrete molecular entities until a more detailed investigation has been made of other physical properties, i.e. electrophoresis, chromatography, etc. and the reactions of these particles with reagents such as cetylpyridinium chloride, a reagent claimed by Buddecke et al. (1963) to produce proteinpolysaccharide particles of lower molecular weight. But it is obvious that before a clear understanding of the role played by these very large macromolecules in the living tissue can be obtained some clear understanding of their nature is required.

7:3:2. PARTICLE SHAPE.

The values computed for $K_s/[\eta]$ in these investigations are all considerably greater than 1.7 (see table 7.2). Creeth and Knight (1965) were unable to offer any explanation for ratios of this magnitude. It is possible that these high values may have real meaning with respect to particle shape and degree of expansion of the macromolecules, but since the choice of models

is limited, because of their intractability to theoretical treatment, no suitable model is yet available to allow interpretation of these observations.

Determination of the axial ratios of these several fractions using equation 7.9 and assigning values of 1.8 and 2/3 respectively for the constants k and ζ gave estimates of J for fractions IA, IIA and IB of less than 1/100 and estimates of 1/8 and 1/5 respectively for fractions IIB and P3 0.75. This would suggest that these molecules behave in solution as extremely flattened oblate spheroids. This prediction would seem very improbable when it is considered that these molecules are composed of highly charged flexible chains.

Most theories concerning the hydrodynamic properties of flexible chain polymers suggest a nearly spherical configuration (J approx. 1) with relatively little flow of solvent through the particle (see for example Kirkwood and Riseman, 1948 and Debye and Bueche, 1948). Thus it would be expected that solutions of macromolecules such as are described here should have $K_s/[\eta]$ ratios near to 1.6, the value obtained by Wales and Van Holde (1954) for random coiled polymers and by other workers for compact spherical molecules (Creeth

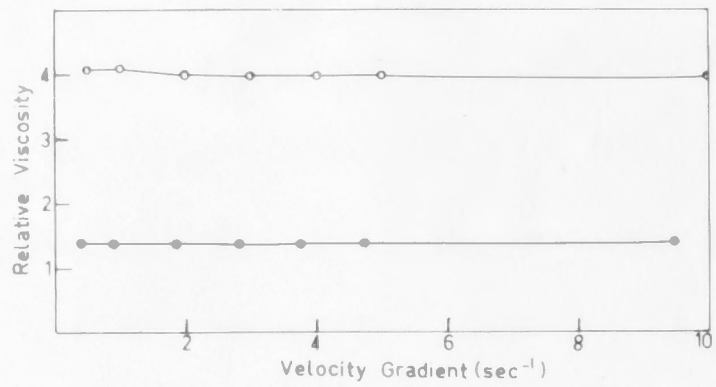

Fig. 7:3. Variation of the relative viscosity with velocity gradient for the proteinpolysaccharide fractions I and II. Fraction I 0.05 g per dl, ● ; fraction II 0.30 g per dl, ○ .

and Knight, 1965). Are then the high values of $K_s/[\eta]$ determined for these preparations not true values?

High values of $K_s/[\eta]$ could arise in two ways

(i) the observed intrinsic viscosity is too small,

(a) because of non-Newtonian behaviour, which would result in the observed viscosities as measured in Ostwald viscometers being too small and/or

(b) because of degradation of the proteinpoly-saccharide material and

(ii) because the value assigned to K_s is too high.

Non-Newtonian characteristics can be demonstrated by measuring the viscosity at different rates of shear using a Couette viscometer of the type described by Preston et al. (1965). In order to establish if the non-Newtonian behaviour was the cause of the high $K_s/[\eta]$ ratio, solutions of fraction I (concentration 0.05 g per dl) and fraction II (concentration 0.30 g per dl) were observed in a Couette viscometer over a velocity gradient ranging from 0.5 to 15.0 sec^{-1}. Fig. 7.3 shows the change in relative viscosity with different rates of shear. It can be seen that both of the preparations gave virtually straight line plots indicating no non-Newtonian behaviour. Further the

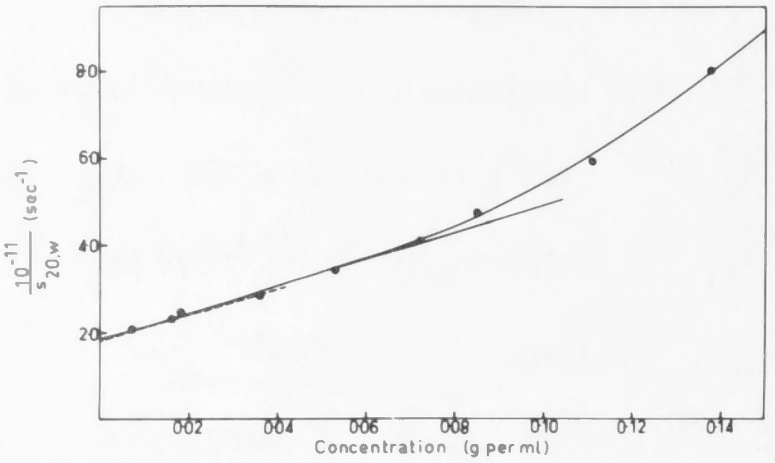

Fig. 7:4. Variation of the reciprocal of the sedimentation coefficient with concentration for fraction IA. Note the progressive upward curvature as the concentration increases.

relative viscosities were in good agreement with those observed in the Ostwald viscometer at much increased rates of shear.

Since the reduced viscosities and sedimentation coefficients for several preparations of each fraction were always in good agreement it was felt that degradative effects were minimal. This then indicates that the high $K_s/[\eta]$ ratio results from an over estimate of K_s.

K_s is composed of two experimentally determined quantities namely, $s^o_{20,w}$ and $d(1/s)/dc$. It would be unlikely that the sedimentation coefficient at infinite dilution is seriously in error. It would be more likely that the error has arisen in the determination of $d(1/s)/dc$.

The 1/s against c plot for fraction IA is shown in Fig. 7.4. It can be seen that at low concentrations (less than approximately 0.07 g per dl) 1/s appears to vary linearly with concentration; however at higher concentrations there is a progressive upward curvature. It is difficult to ascertain precisely at what concentration this curvature commences. If the slope is measured only over the apparently linear portion of the curve then the value assigned to d(1/s)/dc, could be

smaller than that obtained when all points are used.

Ogston and Woods (1954) have observed that there was a similar curvature of the 1/s against c plots when samples of dextran were examined. These authors showed that the 1/s against c plots consisted of two separate linear portions connected by a curved portion. They identified the linear portion with the smaller slope with a concentration range where the solute existed as separate particles and the linear portion of greater slope with a concentration range where the solute particles had become indistinguishable from each other. The values obtained for d(1/s)/dc in this latter region for the dextrans studied was 0.2 to 0.8 x 10^{13} dl per g per sec. These values are similar to those observed in this work and in the work of Luscombe and Phelps (1967a).

From the data given by Ogston and Woods (1954) it is possible to make an estimate of the $K_s/[\eta]$ ratio for each of the two linear portions of the 1/s against c plot. The values estimated for the region of greatest slope and for the region of smaller slope were 3.5 and 1.7 respectively. A similar estimation taken from the data of Luscombe and Phelps (1967a) and Buddecke et al. (1963) gave values nearer 2. This then suggests that

the slope determined in these instances and in our work is that associated with the higher concentration region, or the region where the slope is changing.

Ogston and Woods (1954) have suggested that the concentration range between the two linear portions of the curve occurs at a constant value of the hydrodynamic volume fraction ϕ', where ϕ' is given by

$$\phi' = \frac{c\ V''}{100} \quad \ldots\ldots\ldots\ldots\ldots\ldots \quad (7.10)$$

and c is the concentration in g per ml and V" the effective hydrodynamic volume in ml per g Using this expression and the data given by Ogston and Woods (1954) for dextran samples, the value of ϕ' in the region of concentration connecting the linear portions of the 1/s against c plots can be estimated. A value near 0.06 was obtained.

Assuming that for the connective tissue proteinpolysaccharides the value of ϕ' is also near 0.06 and that the macromolecules occupy approximately spherical domains and knowing a value of $[\eta]$ an estimate can be made of the concentration near where the two linear portions would be expected to link.

According to Ogston (1953)

$$V'' = \frac{100[\eta]}{\xi(J)} \quad \ldots\ldots\ldots\ldots\ldots \quad (7.11)$$

then for a given value of J, the value of V" can be

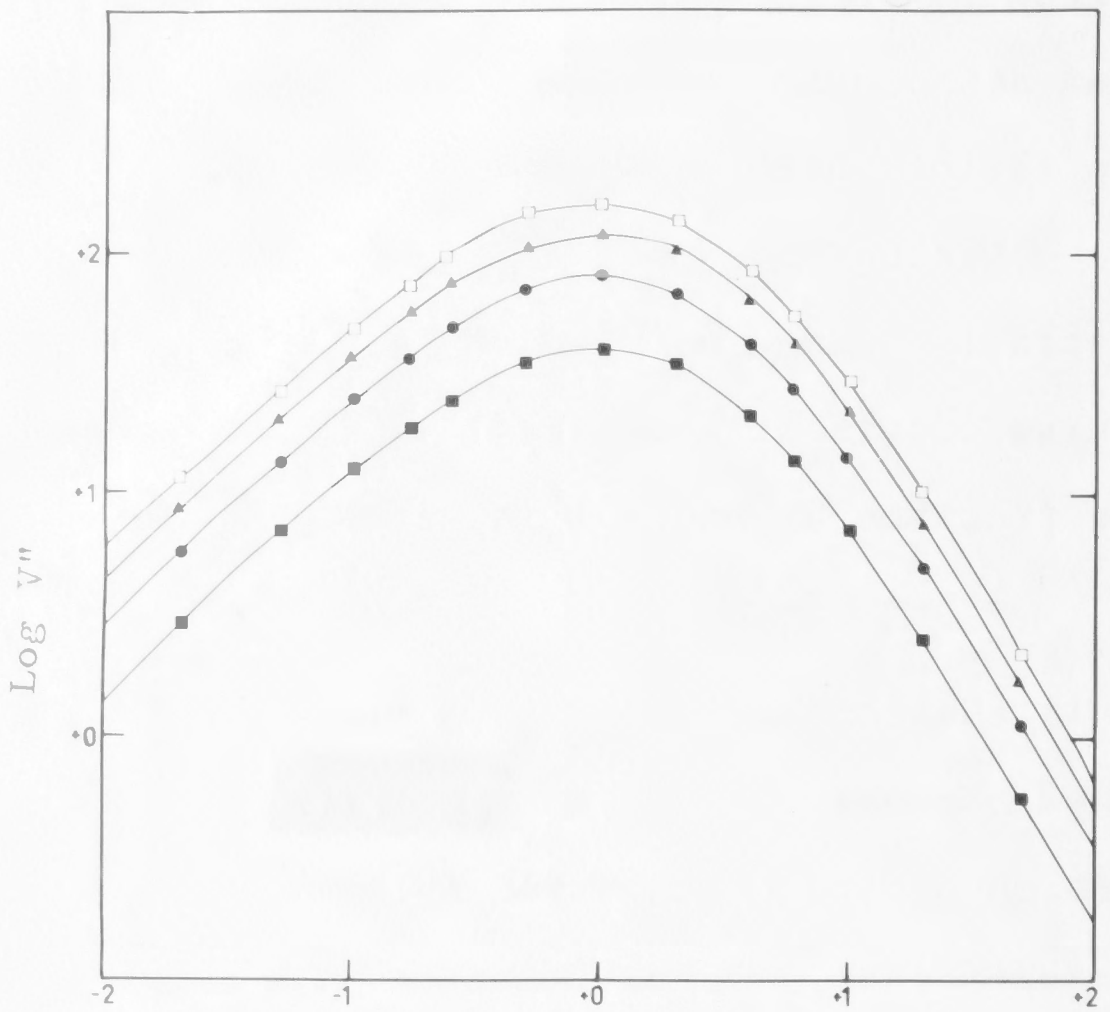

Fig.7:5. Variation of the effective hydrodynamic volume (V", ml per g) with axial ratio for various intrinsic viscosities. ■, 1 dl per g; ●, 2 dl per g; ▲, 3 dl per g; □ 4 , dl per g. Calculated from the equation $V'' = 100[\eta]/\xi(J)$, Ogston (1953).

computed for any intrinsic viscosity. The variation of V"with J for several different intrinsic viscosities is shown in Fig. 7:5. Using this relationship a roughly spherical particle with intrinsic viscosity near 4.0 would have a V" near 150 ml per g and for a particle with [n] near 1.0, a value of approximately 40 ml per g. When these values are substituted into equation 7.10, values of 0.004 and 0.015 g per dl are obtained for the concentrations near to the connecting regions of the suggested linear section of the plots.

Thus in order to obtain data which can be used to correctly estimate $K_s/[n]$, the fractions IA and IIA would have to be observed over the concentration range 0 to 0.004 g per dl. For these types of materials it is experimentally impossible to measure the sedimentation coefficients at these concentrations. However as molecular weight decreases the linear portion with smaller slope extends to higher concentrations and thus it becomes progressively easier to obtain correct values for d(1/s)/dc.

If these assumptions made here are correct either totally or in part then it would be implied that the particles in fractions IA and IIA are interacting with each other so as to appear indistinguishable

even at the very low concentrations studied. This could perhaps explain why the reduced viscosities of the separated fast components were similar to those of the unseparated materials, even though the separated slower components had very much smaller reduced viscosities.

Chapter 8.

General Discussion.

8:1. INTRODUCTION.

The experimental investigations described in the previous chapters have been directed toward

(a) the determination of a method whereby all the proteinpolysaccharides present in any given system could be separated and isolated in such a way that each of the various components would have similar histories of isolation. Justification of such a method is two-fold. Firstly, no one species is isolated at the expense of another, thus the different species can be examined individually and in the presence of each other. Secondly, the comparison of similar proteinpolysaccharides from different connective tissues is permissible.

(b) a comparison of the proteinpolysaccharides from bovine nasal cartilage and similar proteinpolysaccharides from porcine aorta. It was hoped that such an examination would answer one or more of the questions posed in the introductory chapter. Namely, is there a relationship between the molecular weights of the chondroitin sulphate-containing proteinpolysaccharides from different connective tissues ? Is there a fundamental polymeric unit which is common to all the chondroitin sulphate species isolated from different connective tissues and lastly, if such molecular weight units do exist, how and why do they aggregate ?

8:1:1. PREPARATIVE ISOPYCNIC DENSITY GRADIENT CENTRIFUGATION.

The results have indicated the ability of isopycnic density gradient centrifugation to effectively separate mixtures of proteinpolysaccharides into various fractions; it proved completely successful in the separation of a model system of fetuin and a chondroitin sulphate protein preparation and partially successful when applied to the complex mixture of proteinpolysaccharides present in an aqueous extract of aorta. In this latter case the complex was separated into a number of fractions, the components of which appear to be separable either by further density gradient centrifugation or by other techniques of separation.

This technique offers some advantages over the other more frequently used methods of separation, e.g. chrom - atography, electrophoresis, precipitation etc.

The fractionation can be made directly on the crude extract. An added advantage to this is that non-specific protein is removed readily, before or soon after the commencement of centrifugation, since the non-specific proteins have low densities and thus float to the surface of the dense solution. The degradative enzymes remaining in the extract, should then be effectively removed from

that part of the solution containing the glycosamino - glycuronoglycan protein, which have higher densities.

Although the technique has not been effective in fractionating all the proteinpolysaccharide species present in aorta the several components have been so fractionated that the usual separatory techniques, which cannot be applied to crude extracts are applicable. Further the losses of material which accompany the more commonly used methods do not arise in density gradient centrifugation, since all the material within the gradient can be completely recovered. The isolation of such material requires only dialysis to free the solution from CsCl.

It is hoped that the separated components are in forms, near to those of their natural environment, for they have been subjected to conditions no more severe than that of high salt concentrations. Confirmation of the mildness of this technique with proteinpolysaccharide constituents was obtained from observations during density gradient centrifugation of crude extracts of bovine nasal cartilage; the sedimentation and velocity behaviour of the components of bovine nasal cartilage were nearly identical prior to and after density gradient centrifugation.

That this method can be applied to a wide variety of tissue samples has already been demonstrated in these

laboratories. For example, it has been used successfully to fractionate crude extracts of aorta, nasal cartilage, synovial fluid and blood group substances. However the method of density gradient centrifugation is not without limitations.

It allows separation on a density basis only, thus density heterogeneity among the components of a mixture of proteinpolysaccharides reduces the resolving power considerably. Further band width varies inversly as the molecular weight, thus materials of small molecular weight may not be separable even though they may have different densities. Also, polydispersity with respect to molecular weight causes band spreading and reduces resolution by density methods.

The full scope of density gradient centrifugation in the fractionation of proteinpolysaccharides has not been pursued in this work. Mention has been made in the text to the possibility of obtaining better resolution of some of the fractions by repeated density gradients separations. However, the observed behaviour of the connective tissue proteinpolysaccharides in density gradients, suggests possibilities for the wider use of the method. For example, in metabolic studies much interest has been centred on the binding of sulphate to polymeric glycosaminoglycuronoglycan

chains. The addition of sulphate to such chains, should produce an increase in density which might allow a separ - ation of sulphated from unsulphated material in a density gradient. Further, because of the difference in density between protein-containing and protein-free carbohydrate chains, the method may be applicable in the study of interactions between such carbohydrates and proteins.

Finally, the application of the density gradient separation method to the extracts of aortic tissue, has shown that the proteinpolysaccharide composition of this tissue is even more complex than had appeared from earlier studies of the enzyme-degraded tissue (see chapter 1.) A discrete chondroitin sulphate-containing preparation was obtained and characterized, but several other fractions, which had chemical compositions very different from those of the well-known and well-characterized proteinpolysacch- arides, were obtained in an impure form, some of these fractions contained relatively large amounts of hexose. In recent years there has been an active interest in such compounds from the point of view of the structure and function tissues and it is clearly evident that efforts should be made to isolate sufficient quantities of these materials in pure form for characterization.

8:1:2. THE PROTEINPOLYSACCHARIDES OF NASAL CARTILAGE AND AORTA.

The question of the extent to which the conclusions from these investigations may be applied to the physiological function of the proteinpolysaccharides in the ground substance of connective tissue is difficult to ascertain. There can be no doubt that for the proteinpolysaccharides of cartilage at least, the biological stability and consequential significance is directly dependent on the integrity of the protein core. The remarkable finding, that intravaneous injection of the proteolytic enzyme papain into young animals produces a loss of cartilage rigidity (see for example Thomas, 1956), may be explained in terms of the *in situ* degradation of the protein core of the cartilage proteinpolysaccharide. The findings reported here and those reported by other workers suggest that if a fundamental polymeric unit is present in connective tissue proteinpolysaccharides then the amount of protein core material in such units depends upon the source of the connective tissue. Further there is a slight suggestion that molecules containing predominantly chondroitin 6-sulphate have greater amounts of protein core material than whose which contain predominantly chondroitin 4-sulphate (see table 7:3). However as yet, the significance of these observations is not known.

Chondroitin 6-sulphate appears to be a major component, besides hyaluronate, of embryonic tissue (Mathews, 1965), while in new-born and young tissues chondroitin 4-sulphate is present almost exclusively; with increasing age the chondroitin 4-sulphate is steadily replaced with chondroitin 6-sulphate (Meyer 1966a). However before any conclusions can be drawn it is evident that close scutiny must be made of many chondroitin sulphate-containing protein-polysaccharides from different connective tissues, in order to answer questions concerning the function of the 'extra' protein. For example, do the proteins containing chondroitin 6-sulphate always contain more protein material than their chondroitin 4-sulphate counterparts; are the polysaccharide chains in the molecules with the increased amounts of protein core of greater, less or similar length to those in the molecules which contain less protein; are the increased amounts of protein core material simply required to maintain the macromolecular integrity of the particular proteinpoly-saccharide ?

Many workers have described preparations from cartilage, of chondroitin sulphate proteins which have molecular weights greater than 10^7. No such large macromol-ecular entities containing chondroitin sulphate have been reported in other connective tissues. These observations

certainly appear to be true for the proteinpolysaccharides of cartilage and aorta studied here. However, as it was necessary to treat the aortic chondroitin sulphate protein with cetypyridinium chloride, in order that a discrete macromolecular preparation might be obtained, the possibility that this reagent causes some degradation cannot be eliminated. In fact, some preliminary studies with nasal cartilage proteinpolysaccharide have shown that this reagent produces an apparent increase in polydispersity with respect to sedimentation coefficient. Notwithstanding this it seems that the size of the proteinpolysaccharide molecules contained in a particular connective tissue might be a factor in determining the nature of the function of that tissue; for example, in rigid structures such as cartilage, the proteinpolysaccharides contain molecules with very large molecular weights, while in a more elastic tissue such as aorta their molecular weights are smaller. Indeed it has been suggested (chapter 7) that the very large macromolecules interact with each other at extremely low concentrations and this in itself, could be a major contributing factor in the maintenance of the gel-like-structure of the tissues, especially when the material is present at high concentrations. On the other hand, the small molecules interact to a lesser extent at equivalent

concentrations and thus, when present in a elastic tissue, may perform their particular functions without producing excess rigidity

The process by which these large molecules are formed is not known. Meyer (1966a,b) has speculated that it is a process of aggregation in which a basic protein is involved. No protein has yet been isolated that can be unequivocally defined as basic. Further, it was shown here that a glycoprotein material slightly more basic in character than all the other protein components could not be instrumental in aggregation. Is is possible that this glycoprotein material may be necessary to initiate a "polymerization" process, its presence in the preparation after 'polymerization' arising only because of its inability to escape from the network of the macromolecules? Alternatively a species responsible for aggregation might not have been identified. It was observed that when the aortic preparations were centrifuged in CsCl gradients, a thin gel layer which was only difficulty soluble in water was always present at the top of the gradient. It is possible that the insolubility of this gel might result because of the presence of lipid-containing protein material and that this substance may have an effect in the formation of very large macromolecules.

The presence in cartilage of a similar gel fraction was not observed; this detracts from the hypothesis, for only in cartilage are these very large macromolecules observed. Thus, in view of the lack of real evidence to the contrary, it is assumed, for the present, that the types of macromolecules described are representative of the macromolecular species found in the various connective tissues and that they possess individual and characteristic chemical and physical properties.

It was suggested, in the earlier chapters of this thesis, that a careful study of the physical properties of connective tissue proteinpolysaccharides should assist in providing an explanation of the physiological function of these molecules. Much time was spent in developing the method which enabled reasonably discrete macromolecular species to be obtained. Careful studies of the sediment - ation-velocity and viscosity properties of these macro - molecules have been made. Some of the conclusions derived from the experiments have been mentioned earlier in the discussion. One property which has some significance remains to be considered. By assuming that the chondroitin sulphate-containing macromolecules occupy roughly spherical domains (see chapter 7) the effective hydrodymamic volumes (V") vary from about 150 ml per g for the larger molecular

species to about 40 ml per g for the smaller species (perhaps the fundamental unit). This means that in the living tissue these molecules are capable of immobilizing large volumes of water and there is little doubt that this function is important physiologically. Further, the significant difference between the V" values of the large and small molecules could also be important. Thus, in the particular tissue or tissues, it would seem that a smaller amount of large molecular weight proteinpolysaccharide could exert the same water-immobilizing effect as a much larger amount of small molecular weight material. This fact coupled with the special function of the particular connective tissue may determine the size of the proteinpolysaccharide molecule required for the proper physiological functioning of the tissue.

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Appendix 1. Chemical Analysis of the fractions obtained after fraction P1 had been chromatographed on DEAE-cellulose.

FRACTION NOS. POOLED	M NaCl	TOTAL µg PER FRACTION		
		Uronic Acid	Sialic Acid	Hexosamine
0 - 3	0.01	0	50	55
4 - 10	0.01	0	0	0
11 - 30	0.01	0	0	0
31 - 40	0.02	0	0	0
41 - 65	0.05	0	0	13
66 - 93	0.10	0	0	0
94 - 110	0.20	33	52	80
111 - 114	0.20	0	0	0
115 - 130	0.50	306	136	396
131 - 150	0.50	66	18	64
151 - 185	1.00	77	35	98
186 - 212	1.50	0	0	0
213 - 234	2.00	0	0	58
235 - 250	2.00/0.005 HCl	64	0	78

Appendix 2. Values of the functions of J (Ogston, 1953).

J	$\log J$	$\beta \times 10^{-6}$	$\chi(J)$	$\log \frac{\chi^2(J)}{\xi(J)\psi(J)}$	$\log \xi(J)$	$\psi^{\frac{1}{2}}(J)\xi^{\frac{1}{2}}(J)$	$\log \frac{\chi^2(J)}{\psi^4(J)}$	$\log \psi^3(J)$
100	2·000	3·22	5·16	−1·948	2·773	0·338	−1·012	1·828
80	1·903	3·14	4·79	−1·804	2·602	0·352	−0·888	1·689
60	1·778	3·04	4·34	−1·615	2·384	0·368	−0·746	1·516
50	1·699	2·97	4·09	−1·492	2·247	0·380	−0·653	1·408
40	1·602	2·89	3·80	−1·348	2·082	0·398	−0·545	1·278
30	1·477	2·78	3·46	−1·165	1·872	0·421	−0·411	1·117
20	1·301	2·64	3·03	−0·924	1·587	0·456	−0·238	0·901
15	1·176	2·54	2·75	−0·762	1·395	0·480	−0·127	0·754
12	1·079	2·47	2·55	−0·652	1·249	0·504	−0·051	0·648
10	1·000	2·41	2·41	−0·562	1·134	0·520	+0·008	0·565
8	0·903	2·35	2·24	−0·462	1·004	0·541	0·073	0·469
6	0·778	2·28	2·04	−0·349	0·851	0·569	0·146	0·356
4	0·602	2·20	1·81	−0·227	0·669	0·598	0·224	0·218
3	0·477	2·16	1·67	−0·164	0·566	0·610	0·263	0·138
2	0·301	2·13	1·52	−0·121	0·464	0·625	0·288	0·056
1	0	2·12	1·41	−0·097	0·398	0·633	0·301	0
1/2	−0·301	2·12	1·55	−0·094	0·455	0·630	0·308	0·053
1/3	−0·477	2·13	1·78	−0·082	0·535	0·627	0·316	0·137
1/4	−0·602	2·13	2·02	−0·065	0·609	0·625	0·345	0·199
1/6	−0·778	2·14	2·49	−0·043	0·730	0·623	0·366	0·319
1/8	−0·903	2·14	2·95	−0·023	0·826	0·622	0·389	0·414
1/10	−1·000	2·14	3·38	−0·013	0·905	0·622	0·404	0·491
1/12	−1·079	2·14	3·79	−0·002	0·973	0·621	0·414	0·557
1/15	−1·176	2·14	4·35	+0·004	1·058	0·621	0·422	0·641
1/20	−1·301	2·15	5·25	0·018	1·170	0·620	0·436	0·753
1/30	−1·477	2·15	6·85	0·032	1·335	0·620	0·451	0·916
1/40	−1·602	2·15	8·29	0·040	1·452	0·620	0·458	1·034
1/50	−1·699	2·15	9·61	0·047	1·544	0·620	0·463	1·127
1/60	−1·778	2·15	10·85	0·050	1·620	0·620	0·467	1·203
1/80	−1·903	2·15	13·14	0·055	1·742	0·620	0·470	1·325
1/100	−2·000	2·15	15·25	0·057	1·837	0·620	0·472	1·420